Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

JUSTIN L. POWERS, EDITOR, WASHINGTON, D. C.

VOLUME XXXV | *DECEMBER, 1946* | NUMBER 12 | CONSECUTIVE NO. 24

The Colorimetric Assay of Digitoxin by the Modified Raymond Method*

By ROBERT C. ANDERSON and K. K. CHEN†

Digitoxin, when treated in dilute solution with *m*-dinitrobenzene and sodium hydroxide, produces a blue color which can be estimated accurately in a photoelectric colorimeter. The method is particularly useful in the assay of tablets and ampuls of digitoxin. Necessary equipment employed in the test is described. A comparison of the results by this method and those by the U. S. P. cat method is made. The color developed is not specific, but it serves as a quantitative measure if admixture with other cardiac glycosides is not anticipated.

NO SOONER did Gold (1) demonstrate the complete absorption of digitoxin following oral administration in human beings than it became widely used in the treatment of cardiac failures. Additional advantages of digitoxin over galenical preparations are constancy of action because it is a single chemical entity, and absence of constituents irritating to the stomach, so that nausea and vomiting are less apt to occur. Besides, this development represents a phase of medical progress, since more and more active principles have replaced the extractives of plants or animal organs from which the former are isolated.

The assay of digitoxin becomes a new problem. Obviously, the determination of physical constants, such as the melting point and optical rotation, is not sufficient to establish its purity and potency. To insure uniformity of the product, the U. S. P. biological assay by the cat method (2) is undoubtedly the most reliable. Unfortunately, this method is not only costly and time-consuming, but it is often handicapped by a shortage of cats. A chemical or colorimetric method is highly desired, particularly for checking the intermediate steps of manufacture, such as need of recrystallization and assay of the tablet granulations. Bell and Krantz (3) have already elaborated a colorimetric method based on the Baljet reaction (4), and have advocated its use in assaying digitoxin.

During the last few years, we have been

* Received Sept. 4, 1946, from the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis 6, Ind.
▸ Presented to the Scientific Section, A. PH. A., Pittsburgh meeting, August, 1946.
† We are indebted to Messrs. Chester C. Hargreaves and William T. Winchester for their work on the cat assays; also to Mr. Fred A. Rice, Jr., for his assistance, and to Mr. E. Brown Robbins for his statistical help and advice.

investigating the Raymond reaction of *m*-dinitrobenzene (5, 6) with digitoxin. The ultimate color is blue and is easy to measure and compare with that of the standard, although it disappears quickly. The color reaction is believed to be caused by the lactone ring of the glycoside. It has advantages and disadvantages similar to those of the Bell–Krantz method. Numerous comparisons were made with the Bell–Krantz, U. S. P. biological, and the Raymond methods. The majority of the lots of digitoxin were manufactured in our own plant. One specimen was generously supplied to us by Dr. Walter A. Jacobs, Rockefeller Institute for Medical Research, New York City; and another by Professor R. P. Linstead, now Director of the Chemical Research Laboratory, Teddington, England, through the courtesy of Dr. Otto Krayer, Harvard Medical School, Boston.

EXPERIMENTAL

Procedure.—The reagents needed are a 1% solution of *m*-dinitrobenzene in absolute ethanol and a 20% solution of sodium hydroxide. For the test, 1 cc. of *m*-dinitrobenzene solution is added to 10 cc. of dilute digitoxin solution in approximately 47.5% ethanol and placed in an ice bath. After five to ten minutes, 2 cc. of sodium hydroxide solution is added and the mixture returned to the ice bath after thorough mixing. The indigo-blue color produced is read exactly five minutes after the addition of sodium hydroxide. For this test, a Cenco–Sheard–Sanford "Photelometer," a water blank, and No. C orange filter were employed. A portable cooling unit, thermostatically controlled, provided a bath maintained at 0° C. The maximal color develops in five minutes and fades fairly rapidly.

To construct a standard curve, the following dilutions are recommended. Volumes of 0.75 cc., 1.0 cc., and 1.25 cc. of 1:1000 digitoxin solution are diluted to 50 cc. with 47.5% ethanol, so that concentrations of 0.015, 0.020, and 0.025 mg. per cc. are obtained. The blue color in each dilution is produced as described above. The known amounts of digitoxin in the solutions are plotted as ordinates on the equal-division axis of semilogarithmic paper against the "Photelometer" readings as abscissas on the logarithmic scale. Where micro-cells are available, the quantities may be reduced, so that 2 cc. of dilute digitoxin solution, 0.2 cc. of *m*-dinitrobenzene reagent, and 0.4 cc. of sodium hydroxide are used.

To perform the Bell–Krantz test, a Beckman Quartz Spectrophotometer was used in place of the Electrophotometer of the Fisher Scientific Company. The transmissions were compared at 525 mμ. in a 1-cm. cell. The rest of the technique was the same as published.

Results with Unknown Solutions of Digitoxin.—To check the validity of each method, unknown dilutions of a standard lot of digitoxin were prepared and assayed. The results are shown in Table I.

TABLE I.—RESULTS WITH UNKNOWN SOLUTIONS OF DIGITOXIN

Theoretic Concentration %	Observed Concentration Raymond Method, %	Observed Concentration Bell–Krantz Method, %
0.100	0.103	0.105
0.075	0.073	0.078
0.090	0.089	0.092

There is indeed a close agreement between the results of the two methods. The differences between theoretical and observed concentrations are very small. These data clearly indicate that the intensity of color in each method is directly proportional to the concentration of the solution. They attest the accuracy of the curves of known concentrations.

Repeated Tests on the Same Lot of Digitoxin.—For any potent product like digitoxin, it is our practice to set aside a lot to serve as a "House Reference Standard." Such a standard would be assigned a potency of 100%. The potencies of all other lots are based on this figure. Our lot No. 1 was our reference standard. It met all the physical specifications, and by the U. S. P. cat method assayed 2.14 units per mg. The sample has been repeatedly tested colorimetrically by both the Raymond and Bell–Krantz methods, and the results, listed in Table II, have been computed from the predetermined curves. The greatest experimental errors were −3.2% and +2.6%, with the Raymond method; and −4.5% and +4.2%, with the Bell–Krantz method. It is perhaps fair to say that the limits of error by each method is within ±5%.

TABLE II.—RESULTS ON LOT NO. 1[a]

Raymond Method, %	Bell–Krantz Method, %
99.2	99.4
98.7	101.8
102.6	103.9
96.8	95.5
100.7	104.2

[a] "House Reference Standard."

Assay of Experimental and Manufactured Lots.—The relative potencies of 21 lots of digitoxin as determined colorimetrically are compared in Table III. The results by the U. S. P. cat method are also included. Lot No. 1 was the standard for all other lots. It should be noted that the results by the biological method on the one hand and by the two colorimetric methods on the other agree satisfactorily; that is, they are all within the customary

TABLE III.—ASSAY OF VARIOUS LOTS OF DIGITOXIN BY THREE METHODS

Lot No.	Potency: U. S. P. Cat Assay, %	Potency: Raymond Method, %	Potency: Bell–Krantz Method, %
1[a]	100	100	100
2	92	98	96
3	96	94	91
4	89	99	94
5	101	89	98
6	89	91	99
7	106	97	106
8	103	96	104
9	89	84	101
10	96	103	104
11	99	98	102
12	111	103	100
13	106	92	98
14	102	99	102
15	101	97	106
16	94	96	104
17	110	103	105
18	87	99	101
19	96	101	104
20	92	80	98
21	90	82	92

[a] "House Reference Standard."

U. S. P. limits of tolerance of ±20%. Out of 21 lots, the maximal variations by the Raymond method from the biological method are +12% and −14%; and those by the Bell–Krantz method from the biological method are +14% and −11%. The extreme differences between the readings by the two colorimetric methods do not exceed 18% (lot No. 20).

Assay of Tablets.—Thirty tablets, 0.2 mg. digitoxin per tablet, are triturated and transferred to a 60-cc. centrifuge tube. Sixty cc. of 80% ethanol are added and the tube is securely stoppered. It is then placed on an extractor built in our own laboratory, which rotates approximately 20 revolutions per minute. The mixture is allowed to extract overnight and then centrifuged. A volume of 5 cc. of the supernatant liquid is pipetted to a 25-cc volumetric flask and diluted to the mark with 47.5% ethanol. The color is developed as outlined above and theoretically should contain 0.02 mg. per cc. For 0.1-mg. tablets, a volume of 10 cc. of supernatant liquid is diluted to 25 cc., the theoretical quantity of the final dilution remaining 0.02 mg. per cc. Tablet granulations, namely, the powder to be made into tablets, are assayed in a quantity equivalent to the weight of 30 tablets.

Eleven lots of digitoxin tablets of our own make were assayed by the Raymond method. The results as enumerated in Table IV are unquestionably acceptable—without exception.

For comparative purposes, the same lots of tablets were assayed by the Bell–Krantz method. It was soon observed that if absolute methanol was used for extraction the readings would be 15% to 32% too low. Addition of 1 part water to 3 parts methanol, resulting in 75% methanol, readily affects complete recovery of digitoxin. The use of 75% methanol may eliminate any subsequent turbidity as experienced by Bell and Krantz (3), because this dilution will not extract an appreciable amount of the lubricant, such as magnesium stearate. It is also important that extraction should not exceed twenty to thirty minutes, as advised by the authors. Apparently, prolonged extraction, say overnight, would include a sufficient amount of lactose present in the tablets, which will react with picric acid and give rise to a falsely high content of digitoxin. After these precautions are taken, concordant results may be obtained as illustrated in Table IV. In contrast with the Bell–Krantz method, the Raymond method encounters no interference with lactose in spite of prolonged extraction.

It is conceded that neither the Raymond nor the Bell–Krantz method is specific for digitoxin. Similar color reactions take place when other cardiac glycosides are substituted. However, both methods can facilitate the manufacture of digitoxin tablets, provided no interfering ingredient is added. Obviously, the methods are also applicable to ampuls, as we have already tested them several times and found them satisfactory.

TABLE IV.—ASSAY OF DIGITOXIN TABLETS

Tablet Lot No.	Digitoxin Found: Raymond Method, %	Digitoxin Found: Bell–Krantz Method, %
1	96	101
2	98	104
3	103	98
4	95	100
5	93	96
6	100	96
7	95	96
8	100	96
9	106	92
10	101	103
11	103	94

CONCLUSIONS

1. Two colorimetric methods for the assay of digitoxin have been compared. The results on 21 lots of crystalline digitoxin agree in general with those by the U. S. P. cat assay.

2. Eleven lots of digitoxin tablets have been assayed by the same procedures with good agreement.

3. Both methods are accurate, inexpensive, and easily handled, provided certain precautions are observed.

REFERENCES

(1) Gold, H., *Connecticut State Med. J.*, **9**, 193(1945).
(2) "The Pharmacopœia of the United States," Twelfth revision, Mack Printing Co., Easton, Pa., 1942, p. 510.
(3) Bell, F. K., and Krantz, J. C., Jr., *J. Pharmacol.*, **87**, 198(1946).
(4) Baljet, H., *Schweiz. Apoth.-Ztg.*, **56**, 71, 89(1918).
(5) Raymond, W. D., *Analyst*, **63**, 478(1938).
(6) Raymond, W. D., *ibid.*, **64**, 113(1939).

Studies on Pyrogens. II. Some Factors Influencing the Evaluation of Pyrogen Tests*

By HANS MOLITOR, MARY E. GUNDEL, SAMUEL KUNA, and WALTHER H. OTT†

By application of a technique making use of thermocouples for recording body temperatures, more than 6000 pyrogen tests involving over 19,000 rabbits have been conducted. Biometrical analysis of data obtained from these tests and progress toward the development of a quantitative method for the determination of pyrogens using a reference standard and standardized test animals are described.

SINCE the demonstration by Seibert (1, 2) that the occasional elevation of body temperature following intravenous injection of aqueous solutions of medicinal agents, as well as distilled water, is due to pyrogenic substances of bacterial origin rather than to a specific action of the drug itself, increasing attention has been paid to the control and elimination of these contaminants. The combined efforts of numerous investigators in government and industrial laboratories (3, 4) and in hospitals (5), coordinated by the Sub-Committee on Bioassays of the Revision Committee of the U. S. Pharmacopœia, have greatly advanced our knowledge in this field and have resulted in the institution of an official pyrogen assay in the U. S. Pharmacopœia. This test will detect the presence of pyrogens, but it does not permit their quantitative determination. Such information, although unnecessary for the safe clinical use of the material, is highly desirable for research purposes. The chemist working on the purification and structural identification of pyrogens, the pharmacist trying to develop processes for their removal, the pharmacologist investigating their mode and site of action, and the clinician studying their toxicological significance—all require information as to the actual amounts present. Such a quantitative test will for a long time be based on a bioassay procedure. While chemical test methods have been suggested, such as the permanganate titration for organic matter, they are as yet of no practical value because of their lack of sensitivity and specificity; only after the chemical nature of pyrogens is better known may we expect the development of adequate and specific chemical assays. Even then, however, there will be need for a biological test since already there is ample evidence that different types of microorganisms produce pyrogenic substances which differ widely in their chemical composition (6, 7). There is also some indication that pyrogens of different origin possess different pharmacological and toxicological properties, some of them being capable of producing fever immediately, others only after a certain lag (8), while still others are likely to reduce the body temperature before causing a rise (9).

In a previous communication from this laboratory (10), a technique has been described which makes use of thermocouples for the recording of body temperatures instead of the customary rectal mercury thermometer. This results not only in a considerable saving of time but also in more physiological test conditions since it dispenses with the repeated handling of the test animals. During the last two years more than 6000 pyrogen tests involving over 19,000 rabbits have been conducted in our laboratory with this technique with highly satisfactory results. Biometrical analysis of these data as well as continued research on a quantitative assay procedure has suggested possible improvements in the customary assay which will be presented in the following pages.

It is well known that the accuracy of a biological assay primarily depends upon the following factors: (*a*) a constant and dependable reference standard as a basis for the comparison of the unknown substances; (*b*) a standardized test animal, whose deviations from the norm are small and known;

* Received Sept. 13, 1946, from the Merck Institute for Therapeutic Research, Rahway, N. J.
† The valuable technical assistance of Messrs. Charles F. Butz and Gordon B. Thomas is gratefully acknowledged.

(*c*) a valid biometrical procedure for the evaluation of results.

In spite of the large amount of research which led to the establishment of the presently used official pyrogen assay (11) much remains to be done in order to transform this excellent qualitative method into a quantitative one; indeed, it is doubtful whether it will ever be possible to devise a pyrogen test which in accuracy can favorably compare with such standard procedures as the U. S. P. digitalis, ergot, or insulin tests. However, it should be emphasized again that the shortcomings of the present official assay become manifest only if the test is used for strictly quantitative determinations, a purpose for which it was originally not intended.

One of the greatest obstacles in making this test more quantitative is the difficulty in standardizing the test animal. The rabbit, which at present is universally used for this purpose, possesses a notoriously labile temperature regulation. The wide variations in its body temperature reported by different investigators, ranging from an extreme low of 36.6° (12) to an extreme high of 41° (13) are ample proof of the difficulties involved, even though the reported ranges for the *average* high and low temperatures are considerably narrower, e.g., 38.8–39.7° (14); 39.07–39.85° (13); 39.4–39.9° (15). In addition to the wide differences between individual animals there are also considerable daily and hourly temperature fluctuations in the same animal, largely under the influence of nervous factors.

That the temperature-regulating mechanism should be sensitive to nervous stimuli, such as excitement due to handling, is not surprising, since it is well known that in this species other centrally regulated functions, e.g., water metabolism and vascular reflexes, react similarly to stimuli of this nature (16, 17).

MATERIALS AND METHODS

Male albino rabbits obtained from various sources and weighing 1 to 3 Kg. were used. In general the method of conducting the pyrogen test was similar to that described in the U. S. Pharmacopœia XII. However, in the majority of experiments the animals were kept for the entire duration of the test in specially constructed boxes, and stationary thermocouples were used for recording the rectal temperature, following the technique recently described by one of us (10).

The pyrogen concentrate used as a standard in these tests was prepared from *Pseudomonas aeruginosa* (*bacillus pyocyaneus*) according to the technique of Welch, *et al.* (3). A minimum pyrogenic dose was defined as that amount of the standard which was necessary to cause an average temperature rise of 0.5° in rabbits, when injected intravenously. The amount of our concentrate required to produce this effect was usually 1 cc. per Kg. of a dilution of 1:5000.

Experimental

Previously (10) it has been shown that the body temperature of rabbits which are kept quietly in boxes is generally lower than that of animals disturbed by repeated removal from their cages. However, these experiments were not designed to determine whether or not the consistently lower temperature of undisturbed animals was specifically due to the absence of handling or excitement. It was therefore decided to study the influence of these factors by keeping continuous records of a rabbit's body temperature while exposing the animal to a variety of nervous stimuli.

The iron-constantan thermocouples used were of the flexible, rubber-covered type previously described (10). The recording device used in these tests was designed and constructed by Messrs. Michael Kniazuk and Omer S. Lucier of our laboratory and consisted of a conventional system of a reference thermocouple with a second thermocouple and a reflection type galvanometer as an indicator. In order to obtain a continuous record of the temperature, the galvanometer spot was directed to a photoelectric cell, which was so arranged that it automatically followed the galvanometer deflection and traced its position on a moving-paper chart. A deflection of 2 cm./degree C. was found adequate. The response time of the system depended primarily on the heating time of the thermocouple and movement of the galvanometer. Both were found to be practically negligible for this work.

A tracing typical of the effect of handling and of inserting a rectal thermometer is reproduced in Fig. 1. Insertion of the thermometer usually is followed within sixty to ninety seconds by a temperature rise up to 0.7°. The temperature remains elevated for about ten to thirty minutes after which time it slowly returns to normal.

Figure 2 shows the average temperature curve of a group of rabbits taken in the manner described in our previous paper (10) in animals placed in boxes and, after insertion of rectal thermocouples, left undisturbed for several hours. It can be seen that under these conditions the temperature declined during the thirty to forty-five minutes following insertion of the thermocouple; when the lowest point had been reached a slight increase followed, after which the temperature remained relatively constant.

This "basal" temperature was reached about sixty to ninety minutes after the start and was always considerably below the starting temperature.

These results are in agreement with those obtained with continuous recording of the body temperature. They indicate that temperature readings, taken one to two minutes after the insertion of a thermometer (or thermocouple), are on an average 0.2 to 0.3° higher than the "basal" temperature and that it takes about ten to thirty minutes after each handling before the body temperature has returned to the lower level. Obviously, there are differences among rabbits in the degree to which they react to handling; furthermore, the same rabbit is likely to show a progressive decline in reaction as he becomes used to the procedure.

Since the average basal temperature of undisturbed rabbits is well below that of rabbits tested in the usual manner (10), it was of interest to examine the influence of different starting temperatures on the response to a pyrogenic agent. Just as elevated temperature levels are best suited for the demonstration of an effect of temperature lowering agents, it would be expected that a low starting temperature would favor a temperature increase. An examination of Fig. 3 will show that these expectations materialized. The maximum rise following injection of a pyrogenic material was

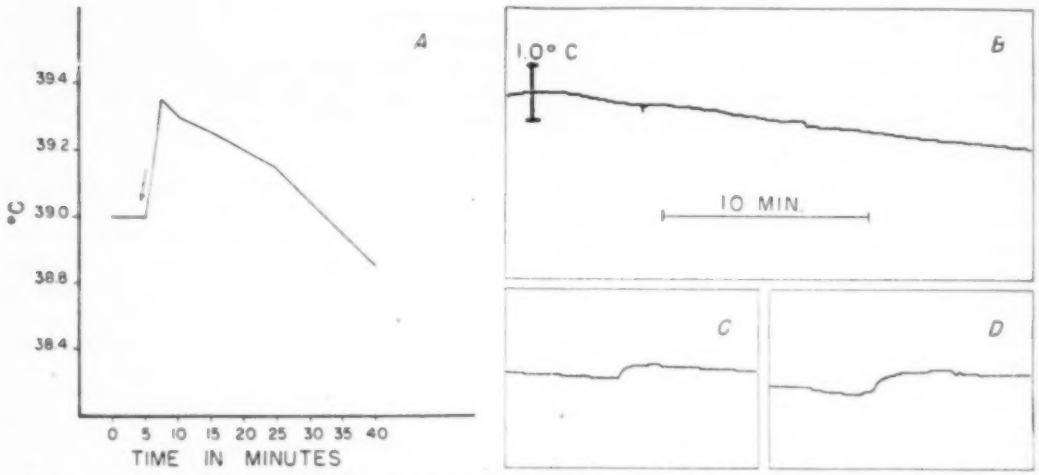


Fig. 1.—Influence of handling on temperature of rabbits. Continuous temperature recordings taken from rectally inserted thermocouples.

A—Temperature rise following mock insertion of a rectal thermometer (with rectal thermocouple remaining in place).

B—Temperature decline resulting from freedom of disturbances. (Rabbit kept in box.)

C and *D*—Effect of slight movements of the rectal thermocouple, simulating insertion of a thermometer

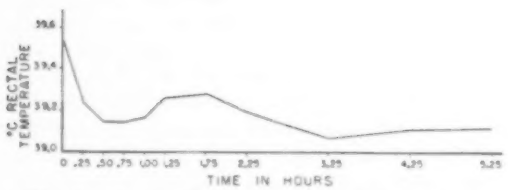


Fig. 2.—Curve showing the change in rectal temperature in a group of 34 rabbits during five hours from the time they were placed in boxes. The average rectal temperature of these rabbits taken with clinical thermometers approximately forty minutes prior to being placed in the boxes was 39.53°.

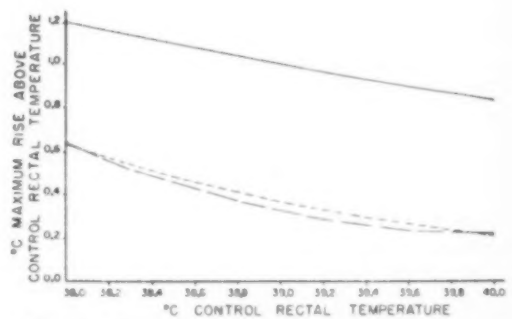


Fig. 3.—Curves showing the relation between control rectal temperature and maximum rise in rectal temperature in noninjected rabbits (dotted line) following intravenous injection of pyrogen-free saline (broken line) or of a standard dosage of pyrogen (solid line). The curve for the pyrogen standard ($Y = 23.69 - 0.9857X + 0.01036X^2$, where Y is maximum rise in ° C. and X is control temperature in ° C.) was constructed from the data of 624 individual tests and represents the average maximum rise observed in rabbits having the corresponding control temperatures. The curve for the pyrogen-free water, $Y = 175.65 - 8.782X + 0.1099X^2$, was calculated from the data of 486 individual tests. The curve for the noninjected rabbits, $Y = 95.91 - 4.695X + 0.05758X^2$, was calculated from data of 731 individual tests. The control temperatures in these 1841 individual tests ranged from 37.1° to 41.6° in a normal frequency distribution with mean ±S. E. of 39.03 ± 0.014°.

inversely proportional to the starting temperature. Thus, the average maximum rise following 4 minimum pyrogenic doses increased from 0.9° at 39.6° to 1.1° at 38.4°. The establishment of an arbitrary limit of temperature variation, beyond which a temperature increase would be regarded as critical, is therefore justified only within a relatively narrow temperature range.

Since animals with a lower starting temperature show a more pronounced reaction to pyrogenic contaminants than those with higher starting temperatures, their use would appear to be preferable. In this case, however, the fixed maximum permissible rise of 0.6°, presently used in the U. S. P. assay, would have to be replaced with a variable limit, in order to obtain physiological equivalent conditions at both high and low initial temperatures. Thus, for example, a temperature rise of 0.8° in the zone of 38.0–38.3° might be approximately equivalent to one of 0.6° at 38.7–39.4° and one of 0.5° at 39.5–40.0°.

Another question is whether the normal fluctuations in body temperature are the same for various initial temperature levels or whether the apparently greater sensitivity of the temperature-regulating mechanism at lower levels is likely to cause wider spontaneous fluctuations at that level. An examination of our data (Fig. 3) indicates that the greater response to pyrogenic stimuli at the lower temperatures is not offset by the greater tendency to "spontaneous" fluctuation. It would therefore seem advantageous to select rabbits with low starting temperatures and to operate under experimental conditions which are likely to produce such a range.

The wide temperature variations reported by previous investigators were obtained under experimental conditions which did not exclude excitement due to handling. It was therefore of interest to study the basal temperature curves of completely undisturbed rabbits, since the elimination of excitement might result in temperatures sufficiently constant to permit quantitative comparisons on the basis of the normal temperature of each individual rabbit. It was found, however, that the daily temperature fluctuations of undisturbed animals, even though much smaller than those obtained with the usual methods of temperature measuring, were nevertheless still too wide to permit an increased accuracy through the standardization of individual animals. Consequently, it seems preferable to base all evaluations on an average group response and to compare this with the response of a group to a pyrogren standard of known potency. The criterion of an average group response is accepted biometrical practice, particularly when it is difficult to standardize individual animals.

While in a more or less qualitative assay designed primarily as a safety control the unfavorable reaction of even a single animal may be sufficient cause for rejection or repetition of the assay, it certainly is inadequate in a quantitative test. We have investigated whether the introduction of the principle of group response would improve the accuracy and convenience of the pyrogen test. The results of this analysis are summarized in Fig. 4 which shows the effect of increased numbers of test animals per group on the accuracy of the test. In this group of

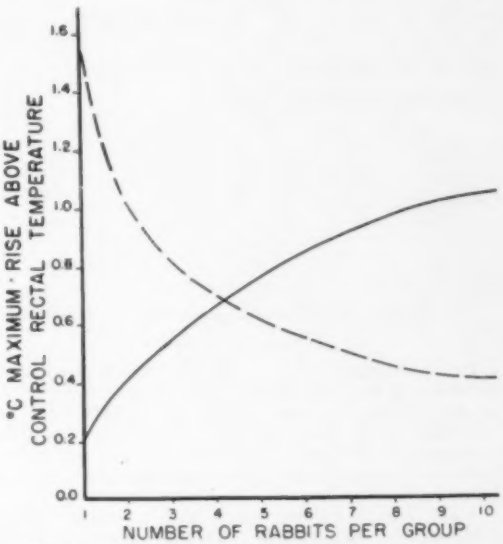


Fig. 4.—Curves showing the tendency for group averages progressively to differ less from the general average maximum rise in rectal temperature as the number of rabbits is increased per group. The average maximum rise in 105 tests following injection of 4 minimum pyrogenic doses per kilogram was 1.20°. The average maximum rise in 83 noninjected rabbits was 0.30°. The individual rises were successively regrouped into groups of different sizes as indicated and the average rises calculated for these groups. The solid curve was drawn through the lowest of the averages thus obtained with the data for the pyrogen-injected animals, and the broken line was drawn through the highest averages found with each of the different sized groups of untreated rabbits.

83 rabbits, the lowest temperature rise following injection of a standardized amount of pyrogen was 0.2° while the greatest rise in temperature of an untreated rabbit when compared with his starting temperature was 1.6°. When the evaluation was based on the average of a group of 3 rabbits, the tendency for the minimal average response to the pyrogen standard to be less than the maximum average fluctuation in a control group of similar size was greatly decreased. In groups of 5 animals or more the lowest average response to the pyrogen standard invariably exceeded the highest average fluctuation in temperature of any control group of similar size. It would therefore appear advantageous to use as a basis for quantitative evaluation the average response of a group of at least 3 to 5 animals and not give undue significance to a high reading occurring in only 1 or 2 animals.

It has been reported by previous authors (18) that there appeared to be little or no quantitative relationship between the dose of the injected pyrogen and the fever response in an individual rabbit.

In our experiments we have found that the individual responses vary so widely that only by consideration of group averages could a direct relationship between the dose of pyrogen and the temperature response be detected. This relation appeared to exist provided the pyrogen dose did not exceed reasonable limits, say, 50 minimal pyrogenic doses. If, however, higher doses were injected, the effect was one of prolongation of the fever rather than of a higher peak reading (Fig. 5).

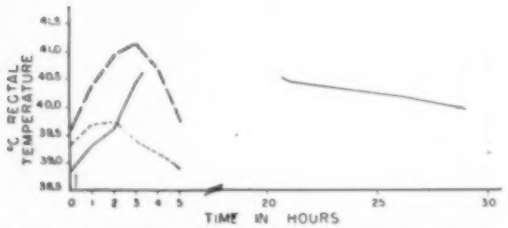


Fig. 5.—Curves showing the change in rectal temperature of rabbits following intravenous injection of standardized amounts of pyrogen solution. The injection was made immediately following the temperature reading at zero time. The lines represent the average temperatures of groups of rabbits as follows:

Solid line: 3 rabbits given 5000 minimum pyrogenic doses. Broken line: 5 rabbits given 80 minimum pyrogenic doses. Dotted line: 5 rabbits given 1 minimum pyrogenic dose.

From a study of the dosage response curve with graded levels of a pyrogen solution it was apparent that the average responses for groups of five rabbits were not uniform enough for satisfactory quantitative use (Experiments 1 to 3, Table I). In one experiment (Experiment 4, Table I), groups of 10 rabbits each were used, and the responses approached linearity when the temperature response was plotted against the logarithm of the dose. These tests were conducted before the influence of starting temperature had been elucidated. However, correction of the individual temperature rises on the basis of the corresponding starting temperature, using the sliding scale mentioned above, did not appreciably affect these results. Later experiments, described below, have indicated that the dose levels were too close together for satisfactory discrimination in these tests.

The first experiment conducted with a group of animals distributed equally on the basis of starting temperature among the different dosages showed a remarkable degree of linearity in the average responses, even though only three rabbits were used in each group (Experiment 5, Table I). These data indicate that a direct relationship might be expected over a wide range of starting temperatures, and that the response to pyrogen is greatest for the low starting temperatures as stated previously (see Fig. 3). Although allocation of the doses to groups equally balanced in advance on the basis of starting temperature is a time-consuming proposition, such a procedure is to be preferred to correction for such differences after the assay has been completed.

TABLE I.—AVERAGE MAXIMUM RISE IN RECTAL TEMPERATURE ABOVE STARTING TEMPERATURE OF RABBITS INJECTED INTRAVENOUSLY WITH GRADED LEVELS OF A STANDARD PYROGENIC SOLUTION

Experiment	Minimum Pyrogenic Doses, No./Kg.	No. Rabbits	Average Starting Temperature, °C.	Average Maximum Rise, °C.
1	1	5	39.32	0.52
	20	5	39.22	1.02
	30	5	39.32	1.48
	40	5	39.60	1.34
	60	5	39.64	1.44
	80	5	39.58	1.64
2	4	5	39.04	1.06
	6	5	39.30	0.96
	8	5	38.90	1.22
3	1	5	39.10	0.26
	2	5	39.10	0.82
	4	5	39.10	0.32
	10	5	39.02	0.72
4	1	10	39.04	0.43
	2	10	39.27	0.64
	4	10	39.19	0.56
	8	10	39.23	0.99
	10	10	39.13	1.10
5a	1.25	3	38.1	1.13
	2.50	3	38.3	1.27
	5.00	3	38.4	1.50
5b	1.25	3	38.7	0.63
	2.50	3	38.8	0.77
	5.00	3	38.8	0.80
5c	1.25	3	39.2	0.60
	2.50	3	39.2	0.77
	5.00	3	39.3	0.93

TABLE II.—AVERAGE TEMPERATURE RESPONSES FOLLOWING INJECTION OF GRADED LEVELS OF A DRY PYROGEN PREPARATION

Experiment	Dose of Pyrogen Preparation, Mg./Kg.	No. Rabbits	Average Starting Temperature, °C.	Average Maximum Rise, °C.
6	0.05	3	39.20	0.57
	0.1	3	38.87	0.87
	0.2	3	39.20	1.20
	0.5	3	39.17	1.60
	1.0	3	38.90	1.97
	2.0	3	39.00	2.13
7	1	3	39.33	1.07
	2	3	39.30	1.23
	8	3	39.40	1.20
8	1	3	39.23	0.83
	2	3	39.10	1.20
	8	3	39.00	1.53
9	0.01	3	38.93	0.23
	0.1	3	39.50	0.47
	1.0	3	39.00	1.07
10	0.05	3	39.03	0.33
	0.5	3	39.03	1.20
	5.0	3	39.03	2.23

Results of preliminary attempts to use a dry preparation of pyrogenic material as a standard are given in Table II. The absolute quantities listed have little significance, since they were largely influenced by the sodium chloride which was used as the dry diluent. It was estimated that 0.05 to 0.1 mg. of this material contained one minimum pyro-

genic dose of pyrogen. The object at this time was to investigate further the nature of the dosage-response curve.

The results (Table II) showed a reasonably satisfactory degree of linearity over a 500-fold change in dosage (0.01 mg. to 5 mg.). In fact it appears that for satisfactory discrimination between successive dosages a dose interval of 5- to 10-fold would be advisable. The average response varied from 0.5° to 1.0° maximum rise per 10-fold increase in dosage. Reference to the data in Table I shows that similar slopes were observed there, but that the dosages were generally too close together for practical purposes.

Robinson and Flusser (7) cited individual responses following injection of graded levels of different pyrogens. Calculated from their data the average responses varied from 0.6° to 0.9° maximum rise per 10-fold increase in dosage, which fall within the range of responses observed above. With this information it appears that application of any of the current biometrical assay methods, such as those described by Knudsen and Randall (18) might be successfully used in a quantitative assay of the pyrogen contents of biological materials.

DISCUSSION

Our experiments demonstrate the great influence of nervous stimuli on the body temperature of rabbits. Animals which are kept undisturbed and from which temperature readings are taken by means of a stationary rectal thermocouple show temperatures on an average 0.2° to 0.3° lower than those observed with the use of repeatedly inserted rectal mercury thermometers. Furthermore, the temperature of animals immediately after removal from their cages is on an average 0.5° higher than sixty to ninety minutes after undisturbed confinement to boxes or stalls. Even after the temperature of an animal has reached its basal level, it will again rise an average of 0.2° to 0.3° under the influence of each handling or insertion of a thermometer. This rise reaches its peak after sixty to ninety seconds and returns to normal within ten to thirty minutes.

An experiment in which the rectal temperature of a group of new, untreated rabbits was measured by means of stationary thermocouples during a period of five weeks showed that the average fluctuation between the temperature at the beginning of each test and that at the end of a three-hour observation period declined from a maximum of approximately 0.4° on the fourth day of the test to less than 0.05° after the twenty-fifth day (Fig. 6). This decline of 0.4° in the maximum fluctuation, due to handling, roughly corresponded to that of another group of new rabbits on which actual pyrogen tests were performed at two- to three-day intervals for a two-week period. In the latter group the maximum response to a standardized dose of pyrogen was 1.0° on the first day as compared to 0.6° on the thirteenth day. It would thus appear that the decline in response to a standard dose of pyrogen which has been reported by previous investigators (19) may, at least in part, be due to the fact that fluctuations of body temperature due to handling and excitement become less pronounced as the animals become accustomed to the experimental procedure. This factor is recognized in the U. S. Pharmacopœia Assay which stipulates that new rabbits must undergo a pretest before the performance of an actual assay and that such a mock test must be repeated when an animal has not been used for more than two weeks. However, our experiments lead us to believe that it requires considerably more than one or two days before an animal becomes completely accustomed to the experimental procedure.

In the light of these findings the temperature-lowering effect of saline and certain bacterial pyrogens reported by Probey and Pittman (9) might be open to a different interpretation. In the case of saline, the lowering of the temperature would simply constitute the normal fall of body temperature in undisturbed rabbits during the first hour of observation. In the case of pyrogens, it might be due to a one- to two-hour lag between the time of injection and the beginning of the pyrogenic effect. The lowering of the temperature observed during the first hour might again, as in the case of saline, reflect the normal temperature of an undisturbed animal which has not yet begun to respond to the injected pyrogen.

In addition to the day-to-day decline in the response to handling there is an immediate temperture rise of 0.2° to 0.3° following the insertion of thermometers, which decreases as the animals become more used to the procedure. This reaction is, of course, completely avoided by the use of stationary rectal thermocouples.

Our experiments with continuous recording show that the temperature rise following handling of a rabbit reaches a maximum within one and one-half

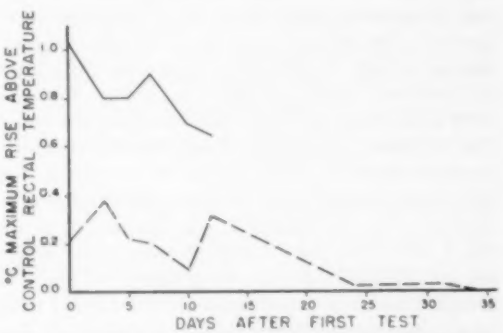


Fig. 6.—Curves showing the decrease in temperature response as additional tests were performed in two groups of rabbits not used prior to the first test in this experiment. In one group of 17 rabbits six tests were conducted in which 4 minimum pyrogenic doses were injected intravenously (solid line). In a different group of 13 rabbits nine tests were performed in the usual manner except that no injections were made (broken line).

to two minutes and declines during the following ten to thirty minutes; on the other hand, it takes between sixty to ninety minutes before the lowest "basal" temperature of an animal has become established.

These observations suggest that in order to avoid readings on a declining slope of the temperature curve one should wait sixty to ninety minutes after the first removal of the animal from its cage before starting an experiment; further, if the (relatively minor) temperature rises due to each insertion of a thermometer are to be avoided, a stationary rectal temperature measuring device should be used.

The technique just described results in a generally lower average starting temperature and a relatively greater sensitivity to thermogenic stimuli. However, since the absolute temperature rise after injection of a standard dose of pyrogen is greater at lower and smaller at higher starting temperature levels, it is obvious that the permissible limit of maximum temperature rises after injection of a test sample should be adjusted to the starting temperature. Figure 3 shows that such an adjustment can easily be made.

These modifications in technique render pyrogen tests more sensitive and convenient; they do not, however, improve the quantitative aspects of the assay. In order to accomplish this purpose it is necessary to follow the principles used in other quantitative bioassay procedures, namely, (*a*) comparison of the unknown sample with a known standard and (*b*) use of an average group response as the basis for biometrical evaluation. With these modifications, the presently used technique can readily be made more quantitative.

In order to estimate the pyrogen content of an unknown sample, it would seem advisable to compare the responses following the intravenous injection of, for example, three 4-fold dilutions of the unknown with 1, 4 and 16 minimum pyrogenic doses of the standard. At least 3 to 5 rabbits should be used on each dose level and the average of the individual temperature rises taken as the criterion of response. The directions in the U. S. P. procedure may generally be followed, except that the control temperature should be taken not sooner than one hour after removal of the animals from the cages. The minimum pyrogenic doses equivalent to the responses with the unknown can be estimated from the dosage response curve drawn for the three responses with the standard; or biometrical procedures similar to those described recently by Knudsen and Randall (18) may be applied. In line with accepted bioassay procedure a standard curve must be obtained for each assay.

The selection of a standard pyrogenic substance is not easy. Although it is relatively simple to prepare a concentrate of high pyrogenicity, and such a concentrate, if kept refrigerated or lyophilized and in sealed ampuls, is stable for at least one year (in contrast to some chemically pure pyrogens which are highly unstable), it must be kept in mind that bacterial pyrogens of different origin vary widely in their pharmacological and toxicological properties. This factor has been recognized in the various collaborative studies initiated by the U. S. Pharmacopœia Revision Committee which selected a pyrogen standard consisting of a mixture of five different pyrogens of bacterial origin.

Unfortunately, the term "pyrogen" is often used rather loosely as if it were to designate a single, pharmacologically well-defined agent. Indeed, many investigators and clinicians abroad are still reluctant to pay much attention to pyrogenic contaminants and the U. S. Pharmacopœia is the only one which officially requires pyrogen tests and prescribes a method for this purpose. A thorough chemical, pharmacological, toxicological and clinical study of this entire field would therefore appear in order.

SUMMARY

1. The manipulations concomitant to the insertion of a rectal thermometer in rabbits are likely to cause an immediate rise in body temperature, reaching its peak within sixty to ninety seconds and requiring ten to thirty minutes for its return to normal.

2. The body temperature of rabbits steadily declines on an average of 0.5° during the first sixty to ninety minutes after removal from their cages.

3. The absolute response to a standard dose of pyrogens is greater at lower than at higher body temperatures.

4. The pyrogen test can be made more quantitative if the average temperature response of the group of rabbits is used as basis for evaluations.

5. The data indicate a linear relationship between the average maximum temperature rise and the logarithm of the dose of pyrogen.

6. The conduct of a quantitative test should involve comparison with a reference standard at several dose levels.

REFERENCES

(1) Seibert, Florence B., *Am. J. Physiol.*, **67,** 90(1923).
(2) *Ibid.*, **71,** 621(1925); *op. cit.*, p. 652.
(3) Welch, H., Calvery, H. O., McClosky, W. T., and Price, C. W., THIS JOURNAL, **32,** 65(1943).
(4) McClosky, W. T., Price, C. W., van Winkle, W. Welch, H., Calvery, H. O., *ibid.*, **32,** 69(1943).
(5) Co Tui, F. W., and Wright, A. M., *Ann. Surg.*, **116,** 412(1942); THIS JOURNAL, *Pract. Pharm. Ed.*, **5,** 60(1944); *Proc. Soc. Exptl. Biol. Med.*, **42,** 549(1939); *ibid.*, **49,** 320 (1942).
(6) Co Tui, F. W., and Schrift, M. H., *J. Lab. Clin. Med.*, **27,** 569(1942).
(7) Robinson, E. S., and Flusser, A B., *J. Biol. Chem.*, **153,** 529(1944).
(8) Hort, Edward C., and Penfold, W. J., *Brit. Med. Soc.*, **16,** 1589(1911).
(9) Probey, T. F., and Pittman, M., *J. Bact.*, **50,** 397 (1945).

(10) Kuna, S., Edison, A. O., and Butz, C., THIS JOURNAL, 35, 59(1946).
(11) "United States Pharmacopœia," Twelfth revision, Mack Printing Company, Easton, Pa., 1942, p. 606.
(12) Winternitz, M. C., and Pratt, J. P., *J. Exptl. Med.*, 12, 7(1910).
(13) Pembly, M. S., *Brit. Med. J.*, 475(1904).
(14) Simpson, S., and Galbraith, J. J., *J. Physiol.*, 33, 225 (1905).
(15) Moore, Lillian M., *Am. J. Physiol.*, 46, 253(1918).
(16) Molitor, H., *Arch. exptl. Path. Pharmakol.*, 113, 172 (1926).
(17) Molitor, H., and Kniazuk, M., *J. Pharmacol.*, 57, 6 (1936).
(18) Knudsen, L. F., and Randall, W. A., *J. Bact.*, 50, 187(1945).
(19) Calvery, H. O., Second U. S. Pharmacopœia Collaborative Study on Pyrogens.

Stable Ascorbic Acid Solution for Parenteral Use*

By JOSEPH L. CIMINERA and PAUL W. WILCOX

The preparation of a stable buffered solution of ascorbic acid suitable for parenteral administration is described. Trisodium phosphate is used to buffer the solution at *p*H 6.0 to 6.5. Manufacture and storage are done in an atmosphere of nitrogen to prevent oxidation. Sterilization is accomplished by heating sealed ampuls for ten minutes at 100°. This is made possible by filling into small-size ampuls (2 cc.) and by incorporating phenol in a concentration of 0.5 per cent.

SINCE ascorbic acid was made available in quantity, many patents concerning the preparation of this chemical in a stable form suitable for parenteral use have issued. The methods described involve the formation of a salt or ester of ascorbic acid in solution and usually include sealing in ampuls under an inert gas with or without the addition of an antioxidant. Some include the preparation of the iron, nickel, cobalt, manganese, cadmium, or zinc salt (1); the magnesium salt (2); the sodium salt in anhydrous alcoholic solution (3); an aqueous solution of the salt of a base of the class consisting of the alkali metals, alkaline-earth metals, ammonia, and the lower alkali-substituted amines, which solution contains a sulfur-containing reducing agent (sulfur dioxide, sulfur-containing acids capable of yielding SO_2, and soluble salts of them) (4); the double calcium salt prepared by the interaction of equimolecular amounts of the calcium salt of quinic acid and the calcium salt of ascorbic acid (5); the quinine and quinidine esters (6); the benzoyl and veratroyl esters (7); the histidine ester (8); the iron, manganese, calcium, bismuth, arsenic, silver, gold, mercury, copper, zinc, aluminum, or tin salt (9); the aliphatic amine esters (10); the ephedrine, amphetamine, or epinephrine ester (11); and the use of thiourea and the methyl and ethyl derivatives of thiourea as antioxidants (12). In addition, the following have appeared on the market at one time or another: sodium ascorbate, monoethanolamine ascorbate, calcium ascorbate, methyl glucamine ascorbate, ascorbic acid crystals, and others.

The above brief review indicates the complexity of the patent picture in this field. Consequently, we have endeavored to develop a stable buffered solution of ascorbic acid that does not infringe any existing U. S. patent, and is acceptable for direct parenteral administration or for addition to intravenous infusions.

To the best of our knowledge, an aqueous solution of ascorbic acid buffered with an alkaline sodium salt has not been described in the U. S. patent literature. After considering several alkaline sodium salts that are available we decided to investigate the possibility of using trisodium phosphate. The use of this compound for buffering ascorbic acid solutions has been described previously (13). However, no details were given for preparing and storing such solutions in a

* Received Aug. 3, 1946, from the Department of Pharmaceutical Research, Medical Research Division, Sharp & Dohme, Inc., Glenolden, Pa.

manner that would guarantee prolonged stability of vitamin C activity. It is the purpose of this paper to present full particulars of a successful procedure.

EXPERIMENTAL

A 10% solution of ascorbic acid was titrated with a solution of trisodium phosphate of the same concentration. Since the solution of ascorbic acid began to discolor at *p*H 7.0 and acquired a yellow-brown color above *p*H 8.0, it was decided to buffer the solution at *p*H 6.3, corresponding to the addition $^1/_3$ mole—equivalent of trisodium phosphate. Accordingly a solution of ascorbic acid was prepared as follows:

Per Cc.		
50 mg. + 10%	Ascorbic Acid.....	55 Gm.
60 mg.	Trisodium Phosphate, $Na_3PO_4 \cdot 12H_2O$ (Mallinckrodt Analytical Reagent)....	60 Gm.
0.5%	Phenol, crystals.....	5 Gm.
	Distilled Water, pyrogen-free, *q. s.*.....	1000 cc.

A volume of 900 cc. of freshly distilled pyrogen-free water was heated to boiling. This was saturated with nitrogen while cooling to 30–35°. The ascorbic acid was added and stirring was continued until complete solution occurred. The trisodium phosphate then was added and dissolved. Finally the phenol, previously melted on a water bath, and sufficient freshly distilled pyrogen-free water (saturated with nitrogen) to make 1000 cc. were added. Stirring was continued under nitrogen until solution was complete. The solution was filtered through a sintered glass filter of medium porosity in an atmosphere of nitrogen and then was filled into sterile flame-sealed ampuls of 2-cc. capacity. This was carried out in an atmosphere of nitrogen under pressure and the ampuls were flushed with nitrogen at 5 pounds pressure for at least two seconds *before* and *after* filling. The sealed ampuls were sterilized by immersion in boiling water, allowing the temperature of the water bath to rise *rapidly* to the boiling point again, and maintaining this temperature for *exactly* ten minutes. The ampuls were immediately removed, and quickly cooled by immersion in running water at 20–25°.

Particular care was taken to exclude air and contact with metals during preparation. The ampuls were stored in a cool place protected from light.

The effectiveness of the sterilization procedure was checked by contaminating the solution with *B. subtilis* spores, and testing for sterility after the heat treatment. Ten thousand spores were added to each cubic centimeter of solution. The *B. subtilis* used was a particularly heat-resistant strain that had been lyophilized on powdered talcum. The concentration of spores in the powder was 2,000,000 per gram. Suitable control experiments proved that the organisms in this powder were viable.

All assays were conducted by the titration method, using 2,6-dichlorophenol-indophenol as suggested by Tillmans (14).

RESULTS

Under these conditions, our ampul solution was sterile after the heat treatment and the *p*H was not altered. Manipulation during preparation resulted in a slight loss of ascorbic acid. However, no further loss was encountered after storage for twelve months in the dark at room temperature. The actual figures were as follows:

	Assay	*p*H
Theoretical	55.0 mg./cc.	6.3
Initial assay before sterilization	50.9 mg./cc.	6.27
Assay after sterilization	51.3 mg./cc.	6.25
Assay after 12 months' storage in the dark at room temperature	50.1 mg./cc.	6.22

DISCUSSION

Ascorbic acid and its salts are known to deteriorate in solution, especially in the presence of air, traces of metals such as copper and iron, and light (15). The most characteristic property of ascorbic acid is its strong reducing action in solution and its ease of oxidation, a reaction that is catalyzed by some metals, especially copper (16) and silver (17).

Ascorbic acid in crystalline form is quite stable. Some laboratories have, therefore, resorted to marketing the dry chemical in sterile vials. This procedure is both tedious and costly. Unless the ascorbic acid in this form is dissolved in a buffer solution or in a solution of a substance capable of partially neutralizing it before use, the resulting solution will be quite irritating when it is injected subcutaneously or intramuscularly. This is due mainly to the inherently low *p*H of solutions of ascorbic acid in distilled water, and is a substantial reason for the partial neutralization of ascorbic acid by the preparation of salts or esters, such as those heretofore described.

The basic material we have employed for partial neutralization of ascorbic acid is an analytical reagent grade of trisodium phosphate, $Na_3PO_4.12H_2O$. This salt has several advantages over many of the other compounds that have been used. It is of more definite chemical composition and is more readily handled than is sodium hydroxide. Both sodium bicarbonate and sodium carbonate react with ascorbic acid with the evolution of carbon dioxide, which has to be removed by degassing under vacuum (4). The analytical reagent grade of trisodium phosphate is substantially free of copper, silver, and other metals detrimental to the stability of ascorbic acid. Another virtue of this salt is its high buffer capacity, which is far superior to that of the other compounds mentioned.

As discussed above, our solution of ascorbic acid began to discolor at *p*H 7.0 and acquired a yellow-brown color above *p*H 8.0. This seems to support

the findings of others that solutions of ascorbic acid are oxidized rapidly upon exposure to light and air, particularly when the *p*H approaches 7.0 (18).

In our preparation it was found desirable to buffer the solution to *p*H 6.3 (6.0–6.5) by the addition of 0.5 mole-equivalent of trisodium phosphate. This *p*H is sufficiently high to prevent discomfort at the sight of injection and yet is well below the critical point where ascorbic acid becomes more easily oxidized.

In order to prevent oxidation of ascorbic acid during manipulation and storage, rigid measures were taken to exclude air. The water used first was boiled to remove dissolved gases and then was saturated with nitrogen during cooling. All subsequent manipulations were carried out under nitrogen. The importance of this step cannot be overemphasized.

The solution may be sterilized by filtration through a sterile all-porcelain candle. All candling procedures, however, entail a rigid aseptic technique to ensure sterility. Wherever possible, it is advantageous to sterilize solutions in sealed ampuls by the use of heat.

At one time it was believed that ascorbic acid was heat-labile, but it is now known that the destruction of the vitamin is an oxidative process. Ascorbic acid can be heated for long periods of time without change, provided all contact with oxygen is excluded (19). Since complete absence of oxygen cannot be assured in the manipulations required for mass production, some oxidation can be expected to occur in solutions that are subjected to autoclaving temperatures or even to the milder heat of tyndallization. The application of two basic principles of sterilization will, however, obviate the need for high temperatures and prolonged periods of heating in order to bring about sterility in solutions. The first is concerned with the size of the container. Underwood has shown that the smaller the container the shorter is the heating period required to bring about sterility of solutions (20). The second principle has to do with the effect of increase in temperature on the germicidal power of phenol. Tilley has shown that there is a considerable increase in this power with an increase in temperature (21). We have applied these two principles to our solution and have found that ten minutes' heating at 100° effectively will sterilize our ampuls provided the size is restricted to 2 cc. or less and provided that phenol in a concentration of 0.5% is present in the solution. No loss of ascorbic acid and no discoloration occurred in our solution when it was subjected to this combined treatment.

The method of sterilization probably could be used for ampuls of larger size, but the period of heating might have to be extended. This could easily be determined by trial.

We have described the preparation of a buffered, stable solution of ascorbic acid containing 50 mg. of ascorbic acid in each cc. of solution. Concentrations up to 200 mg./cc. may be prepared similarly, provided the ratio (by weight) of trisodium phosphate to ascorbic acid is maintained at 12:11.

Our solution was added to intravenous infusions such as normal salt solution, Locke–Ringer's solution, 5% dextrose solution, and human blood plasma without evidence of precipitation. The quantity added represented 100 mg. of ascorbic acid per liter of intravenous infusion.

SUMMARY AND CONCLUSIONS

1. A method for preparing a stable, buffered solution of ascorbic acid is presented.

2. Stability is maintained by adherence to rigid anaerobic conditions by the use of nitrogen throughout manufacture and storage.

3. Solutions containing 50 to 200 mg. of ascorbic acid per cubic centimeter may be buffered to *p*H 6.0–6.5 with an analytical reagent grade of trisodium phosphate, $Na_3PO_4 \cdot 12H_2O$. This is accomplished by using 12 parts by weight of trisodium phosphate to 11 parts of ascorbic acid.

4. Sterilization is accomplished by including phenol in a concentration of 0.5 per cent and by heating the sealed ampuls (2 cc.) at 100° for ten minutes and then cooling.

5. A solution so prepared was found to be stable for at least twelve months at room temperature in the dark.

REFERENCES

(1) Pasternack, R., and Regna, P. P., U. S. Pat. 2, 161,651 (1939).
(2) Pasternack, R., and Regna, P. P., U. S. Pat. 2,165,184 (1939).
(3) Elger, F., U. S. Pat. 2,179,978 (1939).
(4) Stuart, E. H., U. S. Pat. 2,187,467 (1940).
(5) Hoffmann–La Roche, Swiss Pat. 200,299 (1938).
(6) Eisenbrand, J., and Seinz, M., U. S. Pat. 2,140,989 (1938).
(7) Warnat, K., U. S. Pat. 2,150,140 (1939).
(8) Elger, F., U. S. Pat. 2,134,246 (1938).
(9) Ruskin, S. L., Canadian Pat. 375,573 (1938).
(10) Volwiler, E. H., and Moore, M. B., U. S. Pat. 2,132,662 (1938).
(11) Ruskin, S. L., U. S. Pat. 2,294,937 (1942).
(12) Gockel, H., U. S. Pat. 2,297,212 (1942).
(13) "The Merck Report," **47,** 8(1938).
(14) Tillmans, J., *Z. Untersuch. Lebensm.*, **54,** 33(1927).
(15) Arcus, C. L., and Zilva, S. S., *Biochem. J.*, **34,** 61 (1940).
(16) Hess, A. F., and Unger, L. J., *Proc. Soc. Exptl. Biol. Med.*, **19,** 119(1921).
(17) Schlemner, F., Bleyer, B., and Cahnmann, H., *Biochem. Z.*, **254,** 187(1932).
(18) "Merck Service Bulletin," "Ascorbic Acid," 1941, p. 46.
(19) Wood, H. C., Jr., and Osol, A., "United States Dispensatory," ed. 23, J. B. Lippincott Co., Philadelphia, 1943.
(20) Underwood, W. B., "A Textbook of Sterilization," ed. 2, The Lakeside Press, R. R. Donnelley & Sons Co., Chicago, 1941, p. 108.
(21) Tilley, F. W., *J. Bact.*, **43,** 521(1942).

Effect of the Anesthetic and the Rate of Injection of Digitalis upon Its Lethal Dose in Cats*,†

By HARALD G. O. HOLCK, KAZUO K. KIMURA, and BARBARA BARTELS‡

In parallel experiments six groups of 12 cats each were anesthetized with urethane and a like number with ether for injection with different dilutions of digitalis according to the U. S. P. XII method. The curves for urethane and for ether were parallel, indicating that the two methods of anesthesia gave results of about the same precision. The smallest lethal dose of digitalis has been calculated to result with 1.76 per cent of tincture of digitalis with either anesthetic. The lethal dose of digitalis was 6.5 per cent greater with urethane than with ether anesthesia; the difference is statistically significant. The slope of the line shows that an error of about 8 per cent exists between the extremes of the 13 to 19 injections permitted by the Pharmacopœia, due to change in concentration of tincture alone.

IN THE DIGITALIS assays of Bliss and Allmark (1), by the cat method, the concentration of the U. S. P. Reference Standard Digitalis Powder (1942) which would yield the minimum lethal dose was not ascertained. Therefore, a major objective of the present work was the establishment of this dose by the inclusion of a sufficient range of concentrations. In view of the reports by Edmunds, Moyer, and Shaw (2) and by Holck, Smith, and Shuler (3) that the fatal dose of digitalis was from 19 to 20 per cent higher with urethane than with ether and the conflicting report by David and Rajamanickam (4) that values for digitalis with urethane were not significantly different from those with ether (only five cats being used with each anesthetic), we included a series of tests to establish the relation between fatal dosages under these two anesthetics for the present reference standard. Because the studies of Chen, *et al.* (5), indicated that more accurate values may be obtained by considering heart weights in addition to body weights, the hearts of all of our cats were weighed. A secondary objective was to determine whether or not our data would confirm the correction factor of 8 per cent established by Bliss and Allmark (1) for values at the extreme limits of the 13 to 19 injections now permitted by the pharmacopœial method.

EXPERIMENTAL

Parallel experiments, each with 72 cats, were used to study the influence of two anesthetics upon the minimum lethal dose of the U. S. P. Reference Standard Powder (1942), urethane and ether being the anesthetics. Injections were made with six different dilutions of the tincture of digitalis prepared according to the U. S. P. XII, using 0.9% sodium chloride solution as the diluent. Starting with the highest dilution, these increased progressively in strength by 41.42%, giving an increase of 100% for each alternate concentration as the series ascended. Although several authors have reported no seasonal variations or trends (6–8), marked variations may occur due to extraneous and unknown causes, such as inability to obtain cats raised under identical conditions (8). To avoid complications from such variations and from changes which might occur during any one day, six cats, forming a group, were injected on the same day, each with a different concentration of 1.60, 2.26, 3.20, 4.53, 6.40, and 9.05% of the tincture of digitalis. The order of testing the concentrations was varied with the aid of 6 × 6 Latin squares as given in the tables of Fisher and Yates (9); in two groups with urethane the order was reversed for two of the cats, owing to accidental circumstances. Ether and urethane were used alternately as the anesthetic in successive groups of six cats. With urethane we employed a 33.3% solution. An initial dose of 1.2 Gm. per Kg., administered intramuscularly, was supplemented with small doses as needed. All of the tests were carried out between June 2, 1944, and September 3,

* Received Aug. 22, 1946, from the Department of Physiology and Pharmacology, College of Pharmacy, University of Nebraska, Lincoln 8, Neb. (H. G. O. H. and K. K. K.), and the Department of Pharmacology, Yale University, School of Medicine, New Haven 6, Conn. (B. B.).

Presented to the Scientific Section of the A. PH. A., Pittsburgh meeting, 1946.

† This project was aided by a grant to the Department of Physiology and Pharmacology, College of Pharmacy, University of Nebraska, from the Nebraska Foundation by courtesy of the Bankers Life Insurance Company, Miller and Paine, the First National Bank, the Midwest Life Insurance Company, the *Nebraska State Journal* and the *Star*, Gold and Company, and the Lincoln Drug Company, all of Lincoln, and by grants to the same department from the Ciba Pharmaceutical Products, Inc., and S. B. Penick and Company; also by a grant from Eli Lilly and Company to the Department of Pharmacology of the School of Medicine, Yale University.

‡ We are indebted to Professor E. Fullerton Cook for supplies of Reference Standard Digitalis Powder, to Dr. C. I. Bliss for consultations on the design of the experiment and for guiding the mathematical analysis, and to Mr. Tom Miya for aid in the tests.

1945, a period of fifteen months. Injections were never in progress in more than 3 cats at any one time, with at least a one-minute interval between dosing any of these.

The general procedure followed that in the U. S. P. XII (10), 1 cc. of the diluted tincture being administered every five minutes into a femoral vein until the heart beat has ceased. Although the U. S. P. allows a fasting range of sixteen to twenty-eight hours, our variations were kept within eighteen to twenty-six hours. No tincture older than twenty-one days was used, although the Pharmacopœial tolerance is thirty days. After death each heart was excised, the blood vessels cut so as to make the cut surface plane with the heart surface, and the pericardium and extraneous fatty tissue removed. The heart was then opened, the blood or blood-clot removed, the cavities rinsed out and any excess water removed with absorbent paper to leave the walls of the heart chambers in about the same condition as the outer surface. To insure more accurate body weights and also to ascertain the error due to this factor, the urine was expressed after each cat had been anesthetized. The number of cubic centimeters of diluted tincture to be administered was based on the initial body weight minus the weight of the urine. The survival time of each cat was recorded, but it has not been considered in the calculations. The cats were distributed among the various groups so as to equalize approximately the length of time they had been held in the laboratory, the proportion of male and female cats per group, and their body weights. While in our care the animals were kept in large, airy cages in a well-ventilated room and on a standard, adequate diet, including milk.

RESULTS AND DISCUSSION

The original data are summarized in Table I. For the sake of completeness we are including both the arithmetic and the geometric values.

Concerning the amount of urine present, we found that 26 of the 72 cats given urethane and 15 of the 72 given ether had no urine which could be expressed. The three highest amounts of urine, expressed as percentage of body weight, were 3.6, 2.1, and 1.8, respectively, with urethane and 2.0, 2.0, and 1.9 with ether. The urine amounted to less than 1% of body weight in 83% of the cats given urethane and in 78% of those which were etherized.

The first stage in the analysis of the results was to compute, for the urethane and ether cats separately, an analysis of covariance in which the dependent variate was the log-dose of digitalis per cat and the two independent variates were the log-body weight and the log-heart weight. After removing the effect of differences between groups and between concentrations of tincture, a partial regression equation was determined from the error row for each anesthetic. In each case the log-body weight accounted for most of the variation between cats but the heart weight provided an additional correction. The reduction in error due to the use of the heart weight as a second measure had a variance ratio of 3.59 for urethane and 5.62 for ether, one just below and the other just above the 5% point of significance. Although somewhat more precise results would be obtained therefore by the use of the double correction for both body weight and heart weight, the gain was insufficient to compensate for the greater complexity of the analysis.

We then determined within concentrations of digitalis, the slope of the regression of log-dose per cat upon the log-weight of cat. The regression coefficients were $b = 1.098 \pm 0.105$ for urethane and 1.054 ± 0.087 for ether. Both differed from a value of 1 by less than the standard error. For this reason it was decided to use the conventional simpler relationship of log-(mg./Kg.) as the measure of response to digitalis in each cat.

The second stage was to compute an analysis of variance for urethane and for ether separately, isolating differences between groups, between doses

TABLE I.—SUMMARY OF RESULTS FROM TESTS WITH U. S. P. REFERENCE STANDARD DIGITALIS (1942) IN 72 CATS UNDER URETHANE AND 72 CATS UNDER ETHER ANESTHESIA[a]

Concentration of Tincture in Per Cent	Mean Wt. in Kg.[b]	Mean Gm. of Urine	Mean No. of Injections	Mean Fatal Dose, Mg./Kg. Geometric	Mean Fatal Dose, Mg./Kg. Arithmetic	Arithmetic Mean Fatal Dose, Mg./Gm. of Heart
			A. Urethane			
9.05	2.68	12	9.8	88.5	89.0	23.2
6.40	2.83	20	12.7	81.2	81.6	20.8
4.53	2.76	9	17.3	77.4	78.1	20.1
3.20	2.73	13	24.0	76.1	76.8	19.4
2.26	2.81	11	30.1	67.2	68.1	17.8
1.60	2.71	19	44.8	70.6	71.7	19.2
			B. Ether			
9.05	2.70	15	9.5	85.2	86.0	23.3
6.40	2.69	20	12.5	79.3	80.0	20.3
4.53	2.72	17	16.3	72.7	73.5	20.0
3.20	2.66	18	21.6	68.9	69.1	17.0
2.26	2.67	13	28.0	63.2	63.4	16.3
1.60	2.67	13	40.5	64.4	64.8	17.5

[a] The cats were injected intravenously at a rate of 1.0 cc. per Kg. every five minutes until cardiac arrest occurred. Each subgroup contains 12 cats.
[b] After expressing the urine.

and their interaction or error as in a randomized block experiment. The variation between doses was divided into a linear term, a quadratic term, and a remainder. If the lethal dose of digitalis depended upon the concentration of the solution of tincture, it should be possible to express the relation between the two as a curve with linear and quadratic terms. One or both terms should be highly significant. If this curve were substantially a straight line over the entire range of concentrations, the quadratic term should not be significant in comparison with the remainder or error. The mean square for the remaining 3 degrees of freedom between doses should approximate the interaction of doses by groups or error. In both cases the quadratic term was larger than the error although not significantly so and the error terms agreed with each other well within expectation.

For these reasons the two series of experiments have been combined into a single analysis of variance as shown in Table II.

They were related similarly to the log-concentration of the injection fluid, a relation which departed but little from a straight line. The design of the experiment in groups increased its precision appreciably.

The mean response at each rate of injection was then plotted on cross-section paper as in Fig. 1. In both cases the lowest concentration of injection fluid gave a higher lethal dose than the next larger concentration. The two series of points paralleled one another as would be expected from the analysis of variance. The data were then examined in respect to two questions.

The first was to determine the concentration of injection fluid which would lead to the minimum dose since this seemed to be bracketed by the observed range. This can be determined from the ratio of the regression coefficients in fitting a quadratic to the three or four observations covering the range which includes the minimal value. In consequence a new curve was computed by least squares from the totals for both urethane and ether anesthesia at the four lowest concentrations of digitalis. The equation was of the form

$$Y = a' + b_1 x + b_2 x^2,$$

where x is the log-concentration of digitalis. The ratio of b_1 to $2b_2$ gives the value of x at which Y is a minimum. In terms of the log-concentration of digitalis solution, this proved to be

$$x_{min} = 0.246 \pm 0.142$$

TABLE II.—ANALYSIS OF VARIANCE OF THE INDIVIDUAL LOG-DOSES OF DIGITALIS

Term	Degrees of Freedom	Mean Square or Variance	Variance Ratio or F	
Urethane x ether	1	0.026923	...	5.08[a]
Between groups of cats	22	0.005303	1.83[a]	1.00
Combined slope, b_c	1	0.239216	82.46[a]	...
Quadratic, combined results	1	0.009249	3.19	...
Comparing urethane and ether				
In slope	1	0.002857	0.98	...
In quadratic curvature	1	0.000110	0.04	...
Variation of means about separate curves for urethane and ether	6	0.002321	0.80	...
Error:	110	0.002901	1.00	...
In urethane experiments	55	0.003260	...	...
In ether experiments	55	0.002542	...	...

[a] Statistically significant, $P < 0.05$.

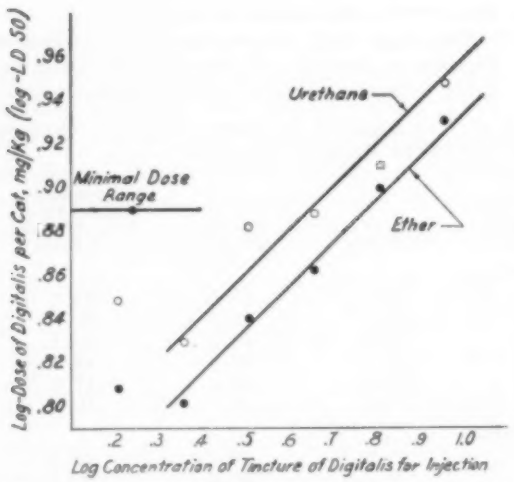


Fig. 1.—A plot of the mean log-doses for data present in Table III.

From the above analysis of variance it is evident that the two anesthetics gave results of about the same precision as judged from the sampling error.

TABLE III.—MEAN LOG-DOSES FOR DATA ANALYZED IN TABLE II AND PLOTTED IN FIG. 1

	Log-Concentration Digitalis	Mean Log-Dose per Cat in Mg./Kg. Urethane	Ether
Plotted points	0.204	0.8489	0.8091
	0.355	0.8275	0.8008
	0.505	0.8816	0.8380
	0.656	0.8888	0.8615
	0.806	0.9098	0.8995
	0.957	0.9469	0.9305
Straight line	0.32	0.8254	0.8005
	1.02	0.9621	0.9372

From the antilog of this result the smallest lethal dose of digitalis in cats was observed when the injection fluid had a concentration of 1.76% of tincture of digitalis. Within limits of one standard deviation this represented 1.27% to 2.45%. Judging from the above analysis of variance, the two forms of anesthesia did not differ significantly in the concentration of digitalis which gave the smallest dose in mg./Kg. If a dilution of the injection fluid were to be selected so as to obtain the minimal lethal dose per cat, an average of 40 instead of the 15 injections in the U. S. P. XII would be required on the basis of these experiments.

The second point at issue was the relative toxicity of digitalis under ether and urethane anesthesia. This could be measured in two ways, from the relative concentrations of injection fluid and from the relative doses at equal rates of injection. For the first procedure the lowest dose was omitted and the results recomputed from the five higher concentrations. The parallel lines shown in the figure have been drawn with this combined slope $b_c = 0.1953 \pm 0.0595$. The horizontal distance between these two lines indicates the relative toxicity in terms of the concentration of digitalis of the urethane-anesthetized cats as compared with those anesthetized with ether. By this definition the log-ratio of toxicity was $M = 0.1272 \pm 0.0494$. In other words, a 34% greater concentration of digitalis was required to give the same lethal dose in cats that had been anesthetized with ether as in those anesthetized with urethane. The second method was to compare the lethal dose with each anesthetic at corresponding concentrations of injection fluid, representing the first row in the analysis of variance. In terms of the log-toxic dose computed from all records, the mean difference was 0.02735 ± 0.01214. At equal concentrations the lethal dose of digitalis was 6.5% greater with urethane than with ether anesthesia, a statistically significant increase.

Another point worthy of note is the close agreement between the slope established in the present studies, $b_c = 0.195 \pm 0.060$ and the one reported by Bliss and Allmark (1), 0.201 ± 0.053, the latter having been calculated from only 4 concentrations. An error of about 8% could arise in the pharmacopœial digitalis assay if the number of injections were to differ from 13 to 19, between the standard and the unknown or test preparations. On the average, the potency of the unknown should be increased about 1.33% for every injection the unknown is lower than the standard or decreased by about the same amount for every injection it is higher.

In these studies the toxic dose with urethane was only 6.5% greater than those with ether, although a difference of 19% to 20% was reported in two previous studies using several samples of digitalis (2, 3). Since the latter values were based on many cats, a question may be raised as to the source of the difference. In each case it apparently held over a wide range of fatal dosage and comparisons were made both with cats receiving the same concentration and with those having about the same survival time. The fact that the administration was continuous rather than intermittent in the earlier studies can hardly explain the difference.

SUMMARY

1. Cats were anesthetized with urethane or ether and injected with different concentrations of digitalis in parallel experiments. The animals were arranged in 24 groups of six, one cat being tested with each of the following concentrations: 1.60, 2.26, 3.20, 4.53, 6.40, and 9.05 per cent of tincture of U. S. P. Reference Digitalis (1942). The data on each cat included its body weight, heart weight, and the lethal dose of drug per Kilogram of cat, using the U. S. P. XII method for assaying digitalis.

2. Although the heart weight provided an additional correction, the gain in accuracy was insufficient to compensate for the greater complexity of the analysis. In the analysis the log-(mg./Kg.) has been used as the measure of response to digitalis in each cat.

3. Curves for urethane and for ether anesthesia relating the logarithm of the lethal dose of digitalis to the log-concentration of injection fluid were parallel. Accordingly, the two experiments were analyzed together, the two methods of anesthesia giving results of about the same precision.

4. With either anesthetic the lowest concentration of injection fluid gave a higher lethal dose than the next larger concentration. The smallest lethal dose of digitalis would be expected with 1.76 per cent of tincture of digitalis, the limits of one standard error being 1.27 and 2.45 per cent. This value did not differ significantly between the two anesthetics.

5. A 34 per cent greater concentration of digitalis was required with the 5 stronger concentrations to give the same lethal dose in cats anesthetized with ether as in those anesthetized with urethane. At equal concentrations of injection fluid, the lethal dose of digitalis was 6.5 per cent greater with urethane than with ether anesthesia, a statistically significant difference.

6. The slope of the line for the 5 stronger concentrations confirmed the value reported by Bliss and Allmark (1) that an error of about 8 per cent in potency could occur due to the effect of concentration, if an assay included the extremes of thirteen and nineteen injections.

7. The error due to inclusion of bladder urine in the body weight was less than 1 per cent in 80 per cent of the cases, and exceeded 2 per cent in only two of the cats.

REFERENCES

(1) Bliss, C. I., and Allmark, M. G., *J. Pharmacol.*, **81**, 378(1944).
(2) Edmunds, C. W., Moyer, C. A., and Shaw, J. R., THIS JOURNAL, **26**, 290(1937).
(3) Holck, H. G. O., Smith, E. L., and Shuler, R. H., *ibid.*, **34**, 90(1945).
(4) David, J. C., and Rajamanickam, N., *Quart. J. Pharm. Pharmacol.*, **7**, 36(1934).
(5) Chen, K. K., Bliss, C. I., and Robbins, E. Brown, *J. Pharmacol.*, **74**, 223(1942).
(6) Wijngaarden, De Lind van, *Arch. exptl. Path. Pharmakol.*, **113**, 40(1926).
(7) Foster, R. H. K., and Van Dyke, H. B., THIS JOURNAL, **22**, 381(1933).
(8) Bliss, C. I., and Hanson, J. C., *ibid.*, **28**, 521(1939).
(9) Fisher, R. A., and Yates, F., "Statistical Tables for Biological, Agricultural and Medical Research," Oliver and Boyd, 1938.
(10) "United States Pharmacopœia," Twelfth revision, Mack Printing Company, Easton, Pa., 1942, p. 510

United States Pharmacopœial Convention

Sixth Annual Financial Report, Covering the Period from May 1, 1945, to April 30, 1946, and Based Upon the Report of the Auditor

STATEMENT OF INCOME AND EXPENSE FOR THE YEAR ENDED APRIL 30, 1946

Income			
Sale of Pharmacopœias:			
Collections		$34,904.75	
Add: Increase in Accounts Receivable			
April 30, 1946	$ 5,411.00		
April 30, 1945	4,868.25	542.75	$35,447.50
Sale of Reference Standards, A. M. A. Articles, etc.:			
Collections		$ 6,188.94	
Add: Increase in Accounts Receivable			
April 30, 1946	$ 916.50		
April 30, 1945	559.50	357.00	
		$ 6,545.94	
Less: Decrease in Collections Pending Remittance to the Treasurer			
April 30, 1945	$ 825.61		
April 30, 1946	719.25	106.36	6,439.58
Interest on Investments:			
Collections		$ 2,431.25	
Add: Increase in Accrued Interest Receivable			
April 30, 1946	$ 830.74		
April 30, 1945	742.19	88.55	
		$ 2,519.80	
Less:			
Amortization of Bond Premiums	$ 157.29		
Purchase of Accured Interest Receivable	18.36	175.65	2,344.15
Miscellaneous Income:			
Collections		$ 180.91	
Add: Net Adjustment of Furnishings and Equipment Account and Reserve		64.75	245.66
Total Income			$44,476.89

Less:				
Expenses				
Publication and Sales Expense		$11,207.25		
Add: Decrease in Inventory				
April 30, 1945	$6,630.34			
April 30, 1946	4,014.59	2,615.75	$13,823.00	
Administration			3,636.92	
Convention			16.99	
Revision		$24,332.98		
Add: Decrease in Inventory Reference Standards				
April 30, 1945	$2,796.18			
April 30, 1946	2,073.02	723.16	25,056.14	
Research			6,177.73	
Stationery and Supplies Inventory Adjustment			111.57	
Provision for Depreciation:				
Building		$ 241.23		
Furnishings and Equipment		202.49	443.72	$49,266.07
Excess of Expense over Income				
For the Year Ended April 30, 1946				$ 4,789.18

STATEMENT OF CASH RECEIPTS AND DISBURSEMENTS (GENERAL ACCOUNT) FOR THE YEAR ENDED APRIL 30, 1946

Cash Receipts and Disbursements				
Cash on Deposit, April 30, 1945				$ 67,368.65
Add:				
Receipts				
Sale of Pharmacopœias			$34,904.75	
Sale of Reference Standards, A. M. A. Articles, etc			6,188.94	
Sale of Securities			15,000.00	
Interest on Investments			2,431.25	
Use of Text by Others			15.00	
Miscellaneous			165.91	58,705.85
				$126,074.50

Less Disbursements	Publications and Sales	Administration	Convention	Revision	Research	Total
Printing and Binding	$11,142.44	.....	.....	.....	.....	$11,142.44
Honoraria	.....	$1,650.00	.....	$10,175.00	.....	11,825.00
Clerical	.....	1,101.06	5.03	6,309.46	144.80	7,560.35
Meetings	.....	320.12	.....	1,522.59	1,052.51	2,895.22
Supplies	.....	45.85	9.00	968.18	353.62	1,376.65
Postage and Telegrams	.....	95.60	2.04	955.59	28.23	1,081.46
Compensation	.....	.....	.....	.....	4,550.04	4,550.04
Headquarters	.....	.....	.....	1,303.39	.....	1,303.39
General	64.81	424.29	0.92	3,098.77	48.53	3,637.32
	$11,207.25	$3,636.92	$16.99	$24,332.98	$6,177.73	$45,371.87

Add:		
Purchase of Securities	$15,000.00	
Purchase of Accrued Interest Receivable	18.36	
Purchase of Furnishings and Equipment	1,406.50	
Building Improvements	1,863.53	$63,660.26
Cash on Deposit, April 30, 1946		$62,414.24

A MORE DETAILED AND SUPPLEMENTARY STATEMENT ON THE REVISION AND RESEARCH EXPENSES

REVISION—MEETINGS

Chairman's Conferences	$ 1,159.11
Hearing, U. S. P. XIII	363.48
	$ 1,522.59

REVISION—SUPPLIES

Stationery	$ 224.40
Mimeograph Supplies	396.00
Miscellaneous Office Expenses	347.78
	$ 968.18

REVISION—SALARIES

Revision Chairman, E. Fullerton Cook	$ 7,375.00
Executive Assistant, Adley B. Nichols	2,800.00
	$10,175.00

REVISION—GENERAL

Reference Standards—Maintenance and Renewals	$ 542.36
Reference Standards—New	2,226.26
Pharmacopœia & Physician Articles—Spanish	182.04
Inter-Society Color Council	25.00
Miscellaneous Expenses	123.11
	$ 3,098.77

BUILDING

Moving	$ 252.84
Maintenance—General and Janitorial	1,050.55
	$ 1,303.39

RESEARCH

Subcommittee	Meetings, Supplies, Postage, Clerical and General	Technical Assistance	
1. Scope	$ 15.70	Dr. Frederick K. Bell, Asst. to Dr. Krantz	$2,500.04
5. Botany & Pharmacognosy	30.39	Kenneth Waters, Asst. to Dr. Beal	$1,800.00
7. Inorganic Chemicals	163.57	Laboratory Assistant—Running Assays and Caring for Animals	$ 250.00
8. Organic Chemicals	4.26		
10. Volatile Oils	30.00		
13. Ointments	318.19		
Penicillin Study	6.40		
Anti-anemia Products Board	195.73		
Endocrine Products Board	737.73		
Sterile Products Board	61.80		
Vitamin Board	31.90		$4,550.04
Digitalis Study	32.02		1,627.69
	$1,627.69		$6,177.73

Total

The Chemical Assay of Penicillin*,†

By MERLIN MUNDELL, HENRY FISCHBACH, and THOMAS E. EBLE

A number of methods for the chemical determination of penicillin have been studied. Two methods, namely, the penicillinase procedure and the method based on the iodometric titration, have been compared with the bio-assay on a large number of samples of penicillin. Data are reported which indicate that the results of either of the chemical methods are more accurate and reproducible than the results of the bioassay. Due to certain technical difficulties inherent in the penicillinase procedure, the authors prefer the iodometric titration to all other methods studied.

FOUR ANTIBIOTICS of the penicillin group have been isolated from commercial products and all have the empirical formula $C_9H_{11}O_4SN_2 \cdot R$ (1). In penicillin F, R is Δ^2 pentenyl ($—CH_2 \cdot CH = CH \cdot CH_2 \cdot CH_3$); R in penicillin G is the benzyl group ($—CH_2 \cdot C_6H_5$); in penicillin X, R is the *p*-hydroxy benzyl group ($—CH_2—C_6H_4OH$); and in penicillin K, R is the n-heptyl group ($—CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_3$).

Penicillin prepared commercially may contain any mixture of the four types of penicillin but most preparations are believed to contain a mixture of penicillins G and K predominately.

These penicillins are known to differ in their activity against certain organisms. For use as a standard, the activity of pure penicillin G against *Staphylococcus aureus in vitro* has arbitrarily been set at 1667 units per milligram. Under similar conditions, penicillins K and X have been shown to have activities of approximately 2300, and 900 units per milligram, respectively, while penicillin F has a potency which has been variously reported to be 1400 to 1900 units per milligram.

The activities *in vivo* experiments do not parallel those determined *in vitro*. Welch, *et al.* (2) have reported that penicillin X is more effective than commercial penicillin in the treatment of gonorrhea on a unit basis. They also report that preliminary studies indicate that penicillin X is three to five times more effective than commercial penicillin in protecting mice against 10,000 lethal doses of pneumococcus. In view of this variation in activity, the ideal method of assay for penicillin would be one which would determine the amount of each of the penicillins present in the preparation. While methods for that purpose have been proposed, they are as yet too time-consuming to be feasible as routine control procedures. Until a simple procedure has been devised for the determination of the amount of each of the various types of penicillin in a mixture, the control of the production of this material should be based on the method of assay which gives the greatest degree of accuracy and the most uniformly reproducible results.

A cup-plate procedure for the assay of penicillin was first proposed by Abraham, *et al.* (3), and was based on the activity of penicillin *in vitro* against *Staph. aureus*. The authors pointed out that, with the use of three plates, the inherent error was probably not greater than ±25%. This method has undergone numerous refinements with the view of increasing the accuracy of the procedure. One modification of this method was adopted as the official method for the assay of penicillin and is described in the Federal Register for September 8, 1945, page 11478. Further study led to the modification of this assay (Federal Register, June 18, 1946, page 3080). Both of these assay techniques have been used in the studies reported herein.

Several chemical methods have been proposed for the assay of penicillin. Of these the following have been investigated in this laboratory: The colorimetric method proposed by Scudi (4), the penicillinase method (5), the alkali inactivation and the H_2O_2

* Received Oct. 28, 1946, from the Chemical Section of the Medical Division, Food and Drug Administration, Federal Security Agency, Washington, D. C.

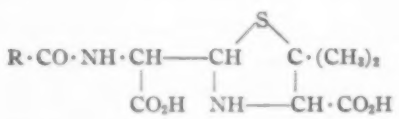
† A portion of this material has been presented by Mr. Eble in partial fulfillment of the requirements for the degree of Master of Science in the Department of Biochemistry, Georgetown University.

inactivation methods proposed by the Chas. Pfizer and Company laboratories (6), the spectrophotometric method proposed by Herriott (7), and the iodometric method proposed by the Squibb Institute for Medical Research (8). The penicillinase method and the iodometric method with some modification seem to give the most consistent results when applied to commercial penicillin products from all manufacturers. Other proposed methods as yet not studied in this laboratory are a colorimetric method proposed by Bristol Laboratories, Inc. (9), and the ninhydrin method (10). This paper is concerned with the results obtained by the penicillinase and the iodometric methods as compared with the results of the two officially adopted procedures referred to above.

EXPERIMENTAL

The Penicillinase Method.—The penicillinase method was developed by Murtaugh and Levy (5). This method is based on the observation of Foster (11) that acid is produced when penicillin solutions are inactivated by penicillinase. Because buffers in the solution interfere with the precise determination of the end point, a highly purified penicillinase preparation is needed. McLuarrie, Liebman, Kluener, and Venosa (12) reported the preparation of such an enzyme which is suitable for use in this assay.

The product of the inactivation of the penicillin is presumed to be penicilloic acid which has the formula:



where R is one of the characterizing groups previously listed. The carboxyl group attached to the thiozolidine ring is identical with the acidic group of penicillin. The other carboxyl, produced by the hydrolysis of the penicillin molecule, is the one measured in the titration. The hydrolysis takes place at the same point in the molecule in all four types of penicillin. The penicillinase method will therefore give the same value for equimolecular quantities of the four penicillins. This has been verified by assaying weighed samples of the crystalline penicillins which were available and results are shown in Table I.

Ten weighed samples of a uniform mixture of sodium sulfate and sodium penicillin G having a potency of 370 units/mg. were dissolved in water and diluted to 25 ml. Aliquots of each of these solutions were taken for duplicate cup-plate and penicillinase assays. The cup-plate assays reported in Table II were obtained by use of the assay described in the Federal Register for September 8, 1945.

TABLE I.—RECOVERY OF CRYSTALLINE PENICILLINS BY THE PENICILLINASE ASSAYS[a]

Sample	Wt. Assayed, Mg.	Wt. Recovered, Mg.	% Recovery
Sodium Penicillin G	5.82	5.77	99.1
	3.31	3.35	101.2
	9.38	9.26	98.7
	7.57	7.53	99.6
Sodium Penicillin F, 175-E-ANWF	4.54	4.83	106.3
	5.68	5.90	103.9
	8.60[b]	8.08	94.0
Sodium Penicillin X, NRRL 1625-7A	6.68	6.27	93.9
	6.68	6.24	93.3
	7.34[b]	6.64	90.5
New Penicillin from culture X1612 (calculated as K)	4.24	4.16	98.1
	5.30	5.04	95.1

[a] The penicillinase used in this work was contributed by the Schenley Research Institute.
[b] Assays made 8 months later than the other assays reported.

TABLE II.—COMPARISON OF THE PENICILLINASE ASSAY AND THE CUP-PLATE ASSAY USING A SAMPLE OF SODIUM PENICILLIN G DILUTED WITH SODIUM SULFATE

Mg. of Mixture Titrated	Units of Penicillin Found[a]	Units/Mg. by Penicillinase Assay[a]	Units/Mg. by Bioassay
36.8	13,450	365	396
	13,450	365	366
38.0	13,900	366	300
	13,750	362	329
38.4	14,200	370	370
	14,200	370	374
39.8	14,600	367	381
	14,600	367	366
36.8	13,700	372	426
	13,850	376	322
40.2	15,350	382	337
	15,050	374	411
42.8	15,350	359	366
	16,000	375	374
41.7	15,200	365	340
	15,350	368	340
37.9	13,750	363	359
	14,200	375	396
38.2	14,100	369	315
	13,850	363	355
	Av.	369	361
Standard Deviation		5.5	31.6
Standard Deviation %		1.5	8.8

[a] Calculated on the basis of 1667 units/mg.

This data shows that for sodium penicillin G the penicillinase and the cup-plate methods give essentially the same values when a sufficiently large number of cup-plate assays are made. The penicillinase method is subject to less variation than the bioassay.

The Iodometric Method.—Although the penicillinase method is well suited for the determination of penicillin in ampuls, a cumbersome extraction is required prior to the inactivation treatment when buffered tablets are encountered. In addition analyses on ointments or troches of low penicillin content are somewhat awkward.

The iodometric method proposed in this paper stems in part from the observations of the Eli Lilly Laboratories (13) and in part from an iodometric procedure suggested by the Squibb Institute of Medical Research (8). The former concluded that sodium penicillin as such is not oxidized by iodine at any *p*H; that the iodine oxidation of sodium penicilloate G or the esters of penicilloate G is probably limited to 9.0 equivalents of iodine per mole. A consumption of 8.5–8.9 equivalents was found by actual experimentation. The latter group, in agreement with the results of the Lilly Laboratories, decided that 2.52 ml. of 0.01 *N* I_2 solution was consumed by the alkaline inactivation product of one milligram of crystalline sodium penicillin G and suggested the conditions for assay.

After consideration of the above information, it was apparent that several time-rate and recovery studies were necessary for the establishing of a fixed procedure. These were carried out at a room temperature of approximately 23°.

Rate of Alkaline Inactivation.—Four-milliliter aliquots of a standard solution of crystalline sodium penicillin G (0.6037 mg./ml.) were added to each of six iodine flasks and 4 ml. of 1.0 *N* NaOH added. Inactivation was allowed to proceed for varying periods of time subsequent to which 4 ml. of 1.1 *N* HCl were added to each flask along with 10 ml. of 0.01 *N* I_2 solution and each permitted to stand for thirty minutes prior to titration with 0.01 *N* $Na_2S_2O_3$.

Rate of Iodine Consumption.—Four-milliliter aliquots of a standard solution of crystalline sodium penicillin G (0.5849 mg./ml.) were added to each of eight iodine flasks and 4 ml. of 1.0 *N* NaOH added and the solution allowed to stand for fifteen minutes. Four milliliters of 1.1 *N* HCl and 10 ml. of 0.01 *N* I_2 solution were then added and time for I_2 consumption was varied from five to forty minutes.

TABLE III.—RATE OF ALKALINE INACTIVATION

No.	No. Min. for Alkaline Inactivation	Ml. 0.01012 *N* I_2 Consumed[a]	% Recovery
1	5	5.85	97.3
2	10	6.00	99.7
3	15	6.05	100.5
4	20	6.05	100.5
5	25	5.95	99.0
6	30	6.05	99.7

[a] The titration of the blank was 9.59 ml. 0.01012 *N* I_2.

It is apparent from the above data that ten to fifteen minutes affords complete inactivation under the prescribed conditions.

TABLE IV.—RATE OF IODINE CONSUMPTION

No.	Time in Minutes for I_2 Consumption	Ml. 0.01012 *N* I_2 Consumed	% Recovered
1	5	5.75	98.3
2	10	5.77	98.6
3	15	5.82	99.4
4	20	5.80	99.1
5	25	5.82	99.4
6	30	5.79	99.0
7	35	5.80	99.0
8	40	5.82	99.4

It appears from Table IV that the reaction between the inactivation product of penicillin and iodine is very rapid, being complete in fifteen minutes.

Finally, it was desirable to ascertain the minimum excess iodine required during the reaction for optimum results. For this purpose 3-ml. aliquots of crystalline penicillin G (0.9004 mg./ml.) were added to each of 11 iodine flasks, inactivated with 3 ml. of 1.0 *N* NaOH for fifteen minutes, 3 ml. of the hydrochloric acid solution, and varying amounts of iodine added. A blank titration was made for each determination. The data are presented in Table V.

Examination of the tabulation below reveals the need of a 25% minimum excess of available iodine in order to achieve a 98% recovery. The

TABLE V.—DETERMINATION OF THE MINIMUM EXCESS IODINE REQUIRED

Exptl. No.	Ml. 0.00970 *N* I_2	Ml. 0.01014 *N* $Na_2S_2O_3$ Blank	Titer After Inactivation, 0.01014 *N* $Na_2S_2O_3$	Ml. I_2 (0.00970 *N*) Consumed	Mg./ml.	% Recovery
1	6.0	5.65	0.0	>5.91	...	...
2	6.5	6.10	0.02	6.36	0.815	90.6
3	7.0	6.70	0.28	6.71	0.861	95.7
4	7.5	7.15	0.71	6.73	0.864	95.9
5	8.0	7.64	1.15	6.78	0.872	97.0
6	9.0	8.64	2.05	6.89	0.884	98.2
7	10.0	9.60	2.98	6.92	0.888	98.6
8	12.5	11.90	5.22	6.98	0.896	99.5
9	15.0	14.61	7.87	7.06	0.906	100.3
10	20.0	19.71	12.66	7.22	0.927	102.6
11	30.0	29.39	22.66	7.04	0.903	100.1

following method, based on these observations, was developed for assaying ampuls of penicillin.

Reagents:

1. 0.01 *N* Iodine Solution
2. 0.01 *N* Sodium Thiosulfate
3. 1 *N* Sodium Hydroxide
4. 1.1 *N* Hydrochloric Acid
5. 1% Starch Solution
6. C. P. Carbon Tetrachloride

Method.—Place 5 ml. aliquots of a solution containing penicillin in a concentration of about 0.6 mg./ml. (1000 units/ml.), in each of two iodine flasks. To one add an equal quantity of 1 *N* NaOH and permit to stand at room temperature for fifteen minutes, and then add 5 ml. of the 1 *N* HCl and 15 ml. of 0.01 *N* I_2. After fifteen minutes titrate the excess iodine with 0.01 *N* sodium thiosulfate, approaching the endpoint with an occasional drop of 1% starch solution as an indicator and completing the titration after the addition of about 5 ml. CCl_4. The latter is particularly advantageous for colored solutions. To the second flask add 15 ml. of the 0.01 *N* I_2 solution and titrate immediately for the "blank determination." Difference in titers ÷ 2.52 = mg. penicillin.

It was known that iodometric titrations are more reproducible when carried out at a low *p*H level (*p*H 2 to 3). Advantage was taken of this fact by using a solution of hydrochloric acid which was approximately 0.1 of an equivalent stronger than the alkali used for inactivation. However, for the blank determination, no adjustment of the *p*H was attempted since this would result in the formation of some degradation products which in turn would react with iodine and yield a correspondingly low value for penicillin potency. It should be noted that among the required reagents only the normality of the sodium thiosulfate solution is critical.

The iodometric assays of various concentrations of different batches of crystalline sodium penicillin G are presented in Table VI.

TABLE VI.—ASSAY OF DIFFERENT BATCHES OF CRYSTALLINE SODIUM PENICILLIN G

No.	Concentrations Used, Mg./Ml.	% Recovery	Ml. 0.01 *N* I_2, Mg.
1	2.66	101.2	2.55
2	0.6004	99.0	2.49
3	5.024	100.3	2.53
4	0.6016	99.9	2.52
5	2.999	101.6	2.56
6	3.001	101.0	2.54
7	2.999	100.8	2.54

The average of the above data indicates that 2.53 ml. of 0.01 *N* iodine solution corresponds to one milligram of sodium penicillin G. This is in good agreement with the 2.52 average reported by the Squibb group. The latter value is used for the basis of the calculations in this report.

A few samples purported to be salts of penicillins F, X, and K were received by this laboratory and previously described under the penicillinase method. These were subjected to the iodometric assay and preliminary results indicate that a similar number of equivalents of iodine react with equimolecular quantities of the respective penicilloates resulting from alkaline inactivation. Although the Squibb group suggested that penicillin X might give abnormally high values, the results in this laboratory do not bear out this opinion.

For the sake of brevity, Table VII exhibits only a few of the typical comparative data obtained for commercial vials of penicillin salts utilizing the Iodometric, the Penicillinase, and the Bioassay methods on the same ampul. The bioassay technique used was that described in the Federal Register for September 8, 1945. Each capital letter represents a different commercial source.

TABLE VII.—COMPARATIVE DATA ON COMMERCIAL VIALS

Manufacturer	% Recovery Based on Manufacturer's Claim Penicillinase[a]	Iodometric[a]	Bioassay
A	121	126	134
	110	109	111
	104	104	100
B	113	113	118
	94	95	94
	93	91	96
C	109	102	110
	113	114	109
	116	117	117
D	99	99	90
	106	107	115
	109	108	95
E	101	99	111
	97	95	106
	96	93	109
F	124	123	110
	107	107	119
	107	110	125
G	103	102	113
	103	101	98
	106	101	98
H	104	104	122
	103	103	108
	104	102	117
I	98	99	105
	94	90	93
	97	100	100
J	92	93	90
	95	99	99

[a] Calculated on the basis of 1667 units/mg.

The above results exhibit good agreement between the chemical methods. The recovery data in each case are based on the claims of the respective manufacturer.

The adoption of an improved bioassay technique (Federal Register, June 18, 1946) made a comparison of the results of this method and of the chemical methods desirable. For this purpose thirty samples of crystalline penicillin G, accurately weighed and varying between 25.5 mg. and 34.5 mg., were diluted separately to 50 cc. One analyst made duplicate penicillinase assays using 10-ml. aliquots of each solution; another analyst made duplicate iodometric assays using 5-ml. aliquots; and the Control

TABLE VIII.—COMPARATIVE ASSAYS OF CRYSTALLINE PENICILLIN G

	Weight of Penicillin, Mg.	% Recoveries Penicillinase	Iodometric	Bioassay
(1)A	32.58	102.4	97.3	99.9
B		101.7	97.5	94.8
(2)A	28.13	102.9	99.5	95.8
B		97.9	99.4	94.2
(3)A	30.26	101.7	97.7	95.2
B		101.3	97.3	98.1
(4)A	26.85	104.9	98.0	93.8
B		103.7	97.7	100.5
(5)A	32.58	102.3	97.4	92.2
B		99.9	97.4	97.4
(6)A	33.47	100.5	99.3	97.7
B		97.8	99.8	102.2
(7)A	29.97	99.7	98.8	96.5
B		99.4	100.0	96.0
(8)A	27.01	99.6	99.2	101.0
B		99.6	99.5	98.0
(9)A	31.05	97.9	97.4	99.0
B		99.5	99.1	93.7
(10)A	30.00	96.2	100.3	99.0
B		98.3	100.7	99.0
(11)A	34.18	99.8	100.0	92.1
B		100.4	100.2	92.1
(12)A	26.23	100.4	100.3	106.3
B		102.2	100.9	105.2
(13)A	25.81	99.8	99.1	98.8
B		100.3	99.6	98.8
(14)A	33.96	100.3	99.9	94.8
B		100.1	100.8	94.5
(15)A	32.07	98.6	99.4	93.5
B		99.2	100.2	92.6
(16)A	30.00	100.2	101.1	98.0
B		99.2	100.8	98.0
(17)A	27.56	100.0	101.7	103.4
B		100.7	101.4	103.4
(18)A	25.95	98.8	101.5	111.0
B		99.0	101.7	109.8
(19)A	25.94	99.6	101.5	104.1
B		99.8	100.0	105.2
(20)A	34.33	98.8	98.5	90.0
B		98.8	98.9	94.4
(21)A	34.37	99.9	100.0	99.5
B		99.5	100.1	99.5
(22)A	34.36	100.7	100.6	97.8
B		99.0	100.7	96.0
(23)A	25.95	99.1	100.5	104.0
B		99.6	100.8	91.3
(24)A	25.95	98.3	99.3	102.9
B		99.0	99.2	105.2
(25)A	30.00	99.1	100.6	98.5
B		98.4	100.4	97.5
(26)A	28.25	97.9	100.8	93.4
B		99.3	100.4	101.9
(27)A	26.46	100.7	100.9	97.5
B		100.3	100.1	95.2
(28)A	32.89	97.7	100.4	96.7
B		97.7	100.0	94.8
(29)A	32.16	98.9	101.9	96.1
B		99.0	99.8	95.6
(30)A	27.10	100.6	101.3	98.5
B		99.8	100.8	100.7
	Av.	99.80	99.82	98.20
Standard Deviation % from the mean		1.5	1.2	4.4
Standard Deviation % from the known weight		1.5	1.2	4.8

Laboratory of the Division of Penicillin Control & Immunology made duplicate assays by the bioassay method referred to above on aliquots of the solutions. The analysts were not told the exact weight of material in the sample until their results were completed. The last ten solutions from samples 26 to 30 were marked so that the analyst did not know which pair were duplicates. The results of this study are tabulated in Table VIII.

In the iodometric titration of samples 1 to 5, inclusive, 10 ml. of 0.01 *N* iodine (representing an excess of about 25%) were used. In samples 6 to 30, inclusive, 15 cc. of iodine solution (representing an excess of about 100%) were used. The improved recoveries due to the use of the larger excess of iodine solution are obvious.

DISCUSSION

The results of the bioassay techniques are determined in units of penicillin, where the solution under investigation is compared with a standard penicillin G solution. Unless the proportion of each of the four types of penicillin in the preparation is known, these results cannot be accurately converted to a weight basis. The results of the chemical methods used in this study are most accurately expressed in milliequivalent weights of penicillin without any reference to the type of penicillin involved. Since the molecular weights of the various known penicillins are of the same order, little error is to be anticipated by converting the results from milliequivalent weights to milligrams by multiplying by the average of the molecular weights of the four penicillins.

Without a knowledge of the proportions of the four penicillins present, however, the results of the chemical assay cannot be accurately translated to the unit basis. Since the results of the bioassay and chemical assays of a preparation of unknown composition cannot be expressed accurately in the same terms, any comparison of the accuracy of the two methods must be made on material of known composition. For this reason the standard deviations of various results were calculated only for those studies in which penicillin G was the only antibiotic present.

As shown in Table II, the standard deviation from the mean for the bioassay described in the Federal Register, September 8, 1945, was found to be 8.8%. Results in

Table VIII show standard deviations of 4.4% from the mean and 4.8% from the known weight when the bioassay described in the Federal Register, June 18, 1946, was used. The same tabulations show a standard deviation from the mean and from the known weight of 1.5% for results by the penicillinase method and a standard deviation of 1.2% for the results of iodometric titration.

A comparison of the two chemical methods shows that each has certain inherent advantages and disadvantages. The penicillinase method requires the use of special equipment and is difficult to apply to some dosage forms of penicillin. While no detailed study has been reported on the action of penicillinase, it is safe to assume that it, like other enzymes, is highly specific and a method based on its action would be specific for penicillin. The iodometric method has the advantage of requiring no special apparatus or reagents. It has been successfully applied to most dosage forms thus far studied and in all probability can be adopted for use in other dosage forms. It is not specific for penicillin, however, and, in the absence of information on the composition of the sample, might require a supplementary analysis by the penicillinase method or a crystallographic study.

We hope, through a collaborative study with a number of manufacturers of penicillin to extend this comparison of the accuracy and reproducibility of the results by bioassay and by iodometric titration.

SUMMARY

A number of chemical methods for the assay of penicillin have been studied. The results by two of the methods, the penicillinase method and the iodometric titration, have been compared with the results of the bioassay. The standard deviation of the data obtained by chemical assay was $^1/_4$ to $^1/_3$ the standard deviation of the results of the bioassay. Of all of the chemical methods tested, the authors prefer the iodometric titration for the determination of penicillin.

REFERENCES

(1) Committee on Medical Research, O. S. R. D., *Science*, **102**, 627–29 (1945).
(2) Welch, H., Putnam, L. E., Randall, W. A., Herwick, R. P., *J. Am. Med. Assoc.*, **126**, 1024 (1944).
(3) Abraham, E. P., Chain, E., Fletcher, C. M., Florey, H. W., Gardner, A. D., Heatley, N. G., Jennings, M. A., *Lancet*, **141**, 177–89, Aug. 16, 1941.
(4) Scudi, J. V., *J. Biol. Chem.*, **164**, 183(1946).
(5) Murtaugh, J. J., and Levy, G. B., *J. Am. Chem. Soc.*, **67**, 1042(1945).
(6) Chas. Pfizer & Co., Inc., report dated Aug. 13, 1945.
(7) Herriott, R. M., *J. Biol. Chem.*, **164**, 725(1946).
(8) Alicino, J. F., *Ind. Eng. Chem., Anal. Ed.* (in press).
(9) Bristol Laboratories, Inc., Private communication.
(10) Allinson, M. J. C., *Proc. Soc. Exptl. Biol. Med.*, **60**, 293(1945).
(11) Foster, J. W., *Science*, **101**, 205(1945).
(12) McQuarrie, E. B., Liebmann, A. J., Kluener, R. G. and Venosa, A. T., *Arch. Biochem.*, **5**, 307(1944).
(13) Eli Lilly and Company, report dated Dec. 16, 1944.

WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

2,3-Dihydroxyanthraquinone
1-Phenyl-3-methyl-5-chloropyrazol
Cinchophen amyl ester
933F (2-Piperidinomethyl-1,4-benzodioxan)
883F (2-Diethylaminoethyl-1,4-benzodioxan)
Crystalline trypsin
2-Phenylbenzopyrone
5,7-Dihydroxy-2-phenylbenzopyrone
2,2′-Dihydroxy-5,5′-dibromobenzil
2,2′-Dihydroxy-3,3′,5,5′-Tetrachlorobenzil
d-Camphor
l-Menthol
3-Chlorotetrahydrofuran
1,1,2,3-Tetrachloroisobutane
1,1,1,2,3-Pentachloroisobutane
1,1,3-Trichloro-2-methyl-1-propene
3-Amino-2,4-dimethylbenzoic acid
2-Methyl-1,3-propylene glycol
1,3-Pentanediol
2-Methyl-1,2-butylene glycol
2-Methyl-1,3-butylene glycol
Pulegone
Thujone
3-Hexen-1-ol

Pharmaceutical Applications of Isopropyl Alcohol. I. As a Solvent in Pharmaceutical Manufacturing*,†

By HENRY M. BURLAGE‡ and DORIS BULLARD HAWKINS

Ten galenical preparations official in the U. S. P. XII and the N. F. VII were prepared with ethyl alcohol and with isopropyl alcohol. Tables of comparison of the specific gravity, *p*H, color, odor, taste, changes on standing, and total extractive of the preparations were compiled.

INTRODUCTION

ISOPROPYL ALCOHOL, dimethyl carbinol, secondary propyl alcohol, CH_3—CHOH—CH_3, was first prepared by Berthelot in 1855 by the reaction of propylene with sulfuric acid and subsequent hydrolysis of the sulfuric acid esters (1). In 1862, Friedel prepared the alcohol by the reduction of acetone (2). Kolbe, in the same year, recognized in isopropyl alcohol the first of the class of secondary alcohols (3). From 1893 to 1900, studies on the compound were made by Zouboff (4), Heinrich (5), and Tischtschenko (6). Others followed in 1904 (7), 1906 (8), 1907 (9), 1909 (10), 1910 (11), 1917 (12), 1920 (13), and 1921 (14, 15).

The first use of the compound in cosmetics appears to have been in 1921 (16). In 1922, the alcohol and glycol were used in the preparation of extracts as a substitute for ethanol (17) and in the same year its pharmacology and applications as a solvent and vehicle were studied (18). Bernhardt in 1922 (19) proposed its use to replace ethyl alcohol in similar concentrations as a disinfectant for the hands. In 1923 the solubilities of certain essential oils in this alcohol were reported (20). It was also recommended in 1926 for use on the skin and clothing (21).

In 1927 and 1928 (22), pharmaceutical applications of the alcohol were reported and Schamelhout's investigations (23) suggested that this compound, because of its similar properties, might be used as a substitute for ethanol in the determination of acid numbers, saponification values of fats, oils, and balsams, and in other tests. In 1938, Ducommun (24) showed that in using a mixture of acetone, isopropyl alcohol, and water in preparing extracts of Cinchona, Belladonna, Nux Vomica, Rhubarb, and Gentian, the active principles were more rapidly extracted than with ethyl alcohol. In 1938, it was used as a solvent in place of ethanol in preparing certain dry extracts of the Swiss Pharmacopœia with similar or improved results (25).

In 1943, its lauric, myristic, and palmitic acid esters were suggested for use as carriers in cosmetic creams, medicaments, perfumes, and flavors (26). In 1944, Lehman and his co-workers (27), using secondary propyl alcohol, prepared Tincture of Digitalis of equivalent or greater potency than one made with the official menstruum. In the preceding year its use in hand soaps (28) and in Liniment of Soft Soap and Liniment of Camphor and Soap was recommended (29). Harris, in his studies on Tincture of Iodine, found that isopropyl alcohol as a solvent yields a product equal to the official preparation in iodine content and antispetic action (30).

Isopropyl alcohol possesses a lower surface tension, a greater fat solvent action, a more rapid killing power on many organisms, is less expensive than ethyl alcohol, and is free from taxation. It has been recommended for use in the preparation of extracts where the solvent is evaporated, in the production of tablet granulations, in pharmaceutical preparations for external use, in diagnostic stains, and in addition has been recently accepted for inclusion in the National Formulary. Therefore a study of its value as a menstruum and solvent in a number of official preparations was undertaken.

* Received Sept. 4, 1946, from the University of North Carolina School of Pharmacy, Chapel Hill, N. C.
Presented to the Scientific Section of the A. PH. A., Pittsburgh meeting, 1946.
† A portion of a thesis submitted as partial fulfillment of the requirements for the M.S. Degree, School of Pharmacy, University of North Carolina.
‡ Professor of Pharmacy, School of Pharmacy, University of North Carolina.

As a basis for the selection of the preparations to be studied, ten which ranked high in frequency of use in a prescription survey recently conducted by Burlage (31) were chosen. These included:

Tincture of Nux Vomica U. S. P. XII.
Tincture of Belladonna U. S. P. XII.
Tincture of Stramonium U. S. P. XII.
Tincture of Hyoscyamus U. S. P. XII.
Camphorated Tincture of Opium U. S. P. XII, First Supplement.
Aromatic Fluidextract of Cascara Sagrada U. S. P. XII.
Elixir of Terpin Hydrate N. F. VII.
Elixir of Terpin Hydrate with Codeine N. F. VII.
Compound Tincture of Cardamon U. S. P. XII.
Elixir of Phenobarbital U. S. P. XII, First Supplement.
Elixir of Three Bromides N. F. VII.
Aromatic Spirit of Ammonia U. S. P. XII.
Compound Elixir of Pepsin N. F. VII.
Elixir of Iron, Quinine, and Strychnine N. F. VII.
Elixir of Five Bromides N. F. VII.
Aromatic Elixir U. S. P. XII.
Fluidextract of Cascara Sagrada U. S. P. XII.

All products except Aromatic Elixir were prepared by official procedures. Aromatic Elixir was manufactured by Shiflett's method (32), which the authors have found to be more satisfactory than the pharmacopœial procedure.

In making these preparations it was found that the following made from isopropyl alcohol, filtered more rapidly than those prepared from ethanol: Camphorated Tincture of Opium, Elixir of Terpin Hydrate, Compound Tincture of Cardamom, and Elixir of Three Bromides; and much more rapidly: Aromatic Elixir and Elixir of Five Bromides.

It was also observed that terpin hydrate dissolved very slowly in isopropyl alcohol and readily in ethanol; that phenobarbital was slightly less readily soluble in isopropyl alcohol than in ethyl alcohol.

EXPERIMENTAL

Physical Constants and Properties of the Products

Specific Gravity.—This constant was determined with a Mohr-Westphal balance at 25° and the values are reported in Table I.

***p*H Values.**—These were determined with a Coleman Electrometer and are reported in Table II.

Physical Properties.—The physical properties studied included (*a*) color of the freshly prepared products (Table III), (*b*) color of the products after storage of nine months or more (Table IV), (*c*) odor (Table V), and (*d*) taste (Table VI).

Determination of Total Extractive

The total extractive of the preparations made by maceration and percolation was determined in the following manner:

Measure 100 cc. of the preparation by means of a transfer pipette into a tared glass evaporating dish which has been previously heated to constant weight in the oven. Allow the sample to evaporate and dry in the air until consecutive weighings vary less than 10 mg. Heat the sample in an oven at 100° for one hour, place in a desiccator for one-half hour and weigh. Repeat the application of heat for one-half hour, cooling and weighing, until a constant weight, varying not more than 0.5 mg. per gram of sample taken, is obtained. These values are shown in Table VII.

It will be noted that the values are less in the case of Tinctures of Belladonna, Hyoscyamus, and Nux Vomica, using the isopropyl alcohol in the menstruum, and noticeably higher in the case of Tincture of Stramonium, Camphorated Tincture of Opium, Compound Tincture of Cardamom and Aromatic Fluidextract of Cascara Sagrada, using the same alcohol.

Assays

All of the tinctures prepared were assayed by the official procedures. In addition, Tincture of Nux Vomica was assayed by the procedure proposed by Burlage and La Rocca (33) whereby the strychnine is separated from the brucine by means of an adsorption column. These values are shown in Table VIII.

The tinctures of Nux Vomica which are not defatted give lower results than the defatted ones by the U. S. P. XII method. This is probably due to the formation of emulsions in the extractions. These emulsions were more frequent, more stable and troublesome in working with samples in which isopropyl alcohol was used, and were obtained even with the defatted tinctures. The undefatted tinctures assayed by the chromatographic procedure, yielded higher values than the defatted tinctures because all of the fatty material was not removed by the adsorption column, thus permitting a probable reaction with the standard alkali.

Isopropyl alcohol yields a tincture of stramonium containing a surprisingly large amount of extractive, but low in total alkaloidal content. It is possible that this low value is due to an interference of some constituent(s) contained in the large amount of extractive.

TABLE I.—SPECIFIC GRAVITIES OF THE PREPARATIONS AND THEIR SOLVENTS

Solvent Concentration	Specific Gravity of Solvent at 25° Ethyl Alcohol	Specific Gravity of Solvent at 25° Isopropyl Alcohol	Preparation	Specific Gravity at 25° Ethyl Alcohol	Specific Gravity at 25° Isopropyl Alcohol	Increase or Decrease Ethyl Alcohol	Increase or Decrease Isopropyl Alcohol
Alcohol: Water, 3:1	0.882	0.867	Tincture of Belladonna	0.891	0.874	0.009	0.007
Alcohol: Water, 3:1	0.882	0.867	Tincture of Hyoscyamus	0.889	0.863	0.007	−0.004
Alcohol: Water, 3:1	0.882	0.867	Tincture of Stramonium	0.892	0.866	0.010	−0.001
Alcohol: Water, 3:1	0.882	0.867	Tincture of Nux Vomica[a]	0.890	0.870	0.008	0.003
Diluted Alcohol: Glycerin, 100:1	0.938	0.930	Camphorated Tincture of Opium	0.965	0.938	0.027	0.008
Diluted Alcohol: Glycerin, 19:1	0.939	0.944	Compound Tincture of Cardamom	0.958	0.939	0.019	−0.005
Alcohol: Water, 1:4	0.977	0.975	Aromatic Fluidextract of Cascara Sagrada	1.112	1.119	0.135	0.144
Alcohol: Water, 1:4	0.977	0.975	Fluidextract of Cascara Sagrada	1.073	1.081	0.096	0.106
...	...	...	Elixir of Phenobarbital	1.160	1.160	...	...
...	...	...	Compound Elixir of Pepsin	1.055	1.056	...	...
...	...	...	Elixir of Terpin Hydrate	1.069	1.077	...	...
...	...	...	Elixir of Terpin Hydrate with Codeine	1.067	1.070	...	...
...	...	...	Elixir of Three Bromides	1.249	1.258	...	...
...	...	...	Aromatic Elixir	1.087	1.083	...	...
...	...	...	Elixir of Five Bromides	1.284	1.301	...	...
...	...	...	Aromatic Spirit of Ammonia	0.898	0.875	...	...
...	...	...	Elixir of Iron, Quinine and Strychnine	1.084	1.097	...	...

[a] The specific gravity of the ethyl alcohol, hydrochloric acid, and distilled water used as a solvent in Tincture of Nux Vomica was 0.883; the isopropyl alcohol, acid, and distilled water mixture had a specific gravity of 0.877.

TABLE II.—*p*H'S OF THE PREPARATIONS AND THEIR SOLVENTS

Preparations	*p*H, Ethyl Alcohol	*p*H, Isopropyl Alcohol	*p*H of Solvent Ethyl Alcohol	*p*H of Solvent Isopropyl Alcohol
Tincture of Belladonna	5.55	5.28	6.05	5.85
Tincture of Hyoscyamus	5.55	5.36	6.05	5.85
Tincture of Stramonium	5.65	5.40	6.05	5.85
Tincture of Nux Vomica[a]	1.78	1.74	0.95	0.98
Camphorated Tincture of Opium	3.95	4.05	6.15	6.05
Compound Tincture of Cardamom	5.30	5.10	5.95	5.80
Aromatic Fluidextract of Cascara	7.80	7.05	6.25	6.05
Fluidextract of Cascara Sagrada	4.60	4.78	6.25	6.05
Elixir of Phenobarbital	4.60	4.70	..	..
Compound Elixir of Pepsin	4.30	4.20	..	..
Elixir of Terpin Hydrate	5.25	4.85	..	..
Elixir of Terpin Hydrate with Codeine	6.80	6.85	..	..
Elixir of Three Bromides	3.36	3.41	..	..
Aromatic Elixir	4.36	4.35	..	..
Elixir of Five Bromides	3.75	3.70	..	..
Aromatic Spirit of Ammonia	9.40	9.40	..	..
Elixir of Iron, Quinine, and Strychnine	3.45	3.34	..	..

[a] The *p*H given above as the solvent for Tincture of Nux Vomica was the acid- alcohol mixture used.

TABLE III.—PHYSICAL PROPERTIES OF THE PREPARATIONS—COLOR

Preparations	Color, Ethyl Alcohol	Color, Isopropyl Alcohol
Tincture of Belladonna	Dark greenish black	Dark greenish black
Tincture of Hyoscyamus	Dark greenish black	Dark greenish black
Tincture of Stramonium	Dark greenish black	Dark greenish black
Tincture of Nux Vomica	Clear deep amber	Clear deep amber
Camphorated Tincture of Opium	Clear brown	Clear brown
Compound Tincture of Cardamom	Dark red	Dark red
Aromatic Fluidextract of Cascara Sagrada	Dark brown	Dark brown
Fluidextract of Cascara Sagrada	Dark brown	Dark brown
Elixir of Phenobarbital	Bright red, cloudy	Clear bright red
Compound Elixir of Pepsin	Clear bright red	Clear bright red
Elixir of Terpin Hydrate	Pale straw	Straw
Elixir of Terpin Hydrate with Codeine	Pale straw	Straw
Elixir of Three Bromides	Clear bright red	Clear bright red
Aromatic Elixir	Colorless	Colorless
Elixir of Five Bromides	Clear dark brown	Clear dark brown
Aromatic Spirit of Ammonia	Clear yellow	Clear yellow
Elixir of I., Q., and S.	Clear bright green	Clear bright green

TABLE IV.—PHYSICAL PROPERTIES OF THE PREPARATIONS—CHANGES ON STANDING

Preparations	Changes, Ethyl Alcohol	Changes, Isopropyl Alcohol
Tincture of Belladonna	No apparent change	Residue on side of container
Tincture of Hyoscyamus	No apparent change	Residue on side of container
Tincture of Stramonium	No apparent change	Residue on side of container
Tincture of Nux Vomica	No apparent change	No apparent change
Camphorated Tincture of Opium	No apparent change	No apparent change
Compound Tincture of Cardamom	Precipitate on top of liquid	No change
Aromatic Fluidextract of Cascara Sagrada	No change	No change
Fluidextract of Cascara Sagrada	No change	No change
Elixir of Phenobarbital	No change	Slight precipitate
Compound Elixir of Pepsin	No change	Light, flocculant precipitate
Elixir of Terpin Hydrate	No change	Changed from pale straw to amber color
Elixir of Terpin Hydrate with Codeine	No change	Changed from pale straw to amber color
Elixir of Three Bromides	No change	No change
Aromatic Elixir	Slightly cloudy	No change
Elixir of Five Bromides	Precipitate	Precipitate
Aromatic spirit of Ammonia	No change	No change
Elixir of Iron, Quinine, and Strychnine	No change	No change

TABLE V.—PHYSICAL PROPERTIES OF THE PREPARATIONS—ODOR

Preparations	Odor, Ethyl Alcohol	Odor, Isopropyl Alcohol
Tincture of Belladonna	Tobacco-like	Tobacco-like
Tincture of Hyoscyamus	Tobacco-like	Tobacco-like
Tincture of Stramonium	Strongly tobacco-like	Strongly tobacco-like
Tincture of Nux Vomica	Slightly sweet	Faint odor of the alcohol
Camphorated Tincture of Opium	Characteristic	Characteristic
Compound Tincture of Cardamom	Cinnamon-like	Cinnamon and of the alcohol
Aromatic Fluidextract of Cascara Sagrada	Heavy sweet odor	Heavy, less sweet odor
Fluidextract of Cascara Sagrada	Bitter, pungent	Bitter, and of the alcohol
Elixir of Phenobarbital	Orange-like	Faint odor of the alcohol
Compound Elixir of Pepsin	Sweet, orange-like	Sweet, faintly of alcohol
Elixir of Terpin Hydrate	Sweet	Faintly of the alcohol
Elixir of Terpin Hydrate with Codeine	Sweet	Faint odor of the alcohol
Elixir of Three Bromides	Odor of benzaldehyde	Odor of benzaldehyde
Aromatic Elixir	Odor of orange	Faint odor of the alcohol
Elixir of Five Bromides	Sickening sweet odor	Less sickening sweet odor
Aromatic Spirit of Ammonia	Characteristic	Characteristic
Elixir of Iron, Quinine, and Strychnine	Orange-like	Less orange-like

TABLE VI.—PHYSICAL PROPERTIES OF THE PREPARATIONS—TASTE

Preparations	Taste, Ethyl Alcohol	Taste, Isopropyl Alcohol
Tincture of Belladonna	Alcoholic	Slightly burning
Tincture of Hyoscyamus	Alcoholic	Slightly burning
Tincture of Stramonium	Strongly tobacco-like	Tobacco-like, burning
Tincture of Nux Vomica	Very bitter	Burning, bitter
Camphorated Tincture of Opium	Aromatic, burning	Aromatic, less burning
Compound Tincture of Cardamom	Aromatic	Slightly burning
Aromatic Fluidextract of Cascara Sagrada	Syrupy, sweet	Syrupy, sweet
Fluidextract of Cascara Sagrada	Very bitter	Very bitter, burning
Elixir of Phenobarbital	Sweet, aromatic	Aromatic, faintly burning
Compound Elixir of Pepsin	Faintly alcoholic	Faintly alcoholic
Elixir of Terpin Hydrate	Sweet, aromatic	Aromatic, faintly burning
Elixir of Terpin Hydrate with Codeine	Slightly bitter	Slightly bitter and burning
Elixir of Three Bromides	Very saline	Less saline
Aromatic Elixir	Aromatic	Slightly burning
Elixir of Five Bromides	Very saline	Less saline
Aromatic Spirit of Ammonia	Burning	Burning
Elixir of Iron, Quinine, and Strychnine	Very bitter	Bitter

TABLE VII.—TOTAL EXTRACTIVE IN THE PREPARATIONS

Preparations	Total Extractive, Gm./100 Cc. Ethyl Alcohol	Total Extractive, Gm./100 Cc. Isopropyl Alcohol	Description of Residue Ethyl Alcohol	Description of Residue Isopropyl Alcohol
Tincture of Belladonna	2.4974	2.4112	Dark green	Black
Tincture of Hyoscyamus	1.8206	1.4379	Light brown	Dark brown-green
Tincture of Stramonium	0.3727	2.5359	Green-brown	Dark brown
Tincture of Nux Vomica	1.7423	1.6859	Brown	Brown
Camphorated Tincture of Opium	8.1509	8.4052	Brown	Brown
Compound Tincture of Cardamom	5.8649	6.7965	Deep red oily liquid	Deep red oily liquid
Aromatic Fluidextract of Cascara Sagrada	34.6987	38.4526	Dark brown	Dark brown

TABLE VIII.—ANALYTICAL VALUES FOR THE TINCTURES

Preparations	Total Extractive Ethyl Alcohol, Gm./100 Cc.	Alkaloids, Ethyl Alcohol, Gm./100 Cc.	Total Extractive Isopropyl Alcohol, Gm./100 Cc.	Alkaloids, Isopropyl Alcohol, Gm./100 Cc.
Tincture of Nux Vomica, U. S. P., undefatted	1.7423	Strychnine 0.11587 0.11566	1.6859	Strychnine 0.09771 0.10293
Tincture of Nux Vomica, U. S. P. XII, defatted	...	Strychnine 0.14736 0.14715	...	Strychnine 0.13399 0.13364
Nux Vomica, chromatographic assay, undefatted	...	Strychnine 0.18480 0.18450	...	Strychnine 0.22338 0.22251
Nux Vomica, chromatographic assay, defatted	...	Strychnine 0.15717 0.15717	...	Strychnine 0.22048 0.22137
Tincture of Belladonna, U. S. P. XII procedure	2.4974	0.03907 0.03964	2.4112	0.03907 0.39940
Tincture of Stramonium, U. S. P. XII assay	0.3727	0.03791 0.03704	2.5359	0.02858 0.02848
Tincture of Hyoscyamus, U. S. P. XII procedure	1.8206	0.00366 0.00360	1.4379	0.00469 0.00466
Camphorated Tincture of Opium, U. S. P. XII procedure	8.1509	0.04994 0.04983 0.04975	8.4052	0.04975 0.04983 0.04966

SUMMARY AND CONCLUSIONS

1. From the standpoint of ease of preparation, isopropyl alcohol is of advantage as a solvent in eight of the preparations studied.

2. The odor of isopropyl alcohol is an objectionable one and is not easily masked; however, benzaldehyde seems to accomplish this quite effectively.

3. Isopropyl alcohol compares favorably with ethanol in preparing Camphorated Tincture of Opium by maceration, and Tincture of Belladonna by percolation. In Tincture of Hyoscyamus, it proved to be a better solvent of the total alkaloids.

4. In the case of the tincture of Nux Vomica, the tincture containing isopropyl alcohol showed less strychnine when assayed by the Pharmacopœial method than that containing ethyl alcohol, but showed a higher strychnine content using the chromatographic method of assay.

5. The alcohol is apparently an unsatisfactory solvent for the preparation of Tincture of Stramonium because of the low yield of alkaloids and the high yield of extractive.

REFERENCES

(1) Berthelot, Marcellin, *Ann. chim. phys.*, **43** (3), 399 (1855); *Jahresber. fortsch. Chem.*, 611(1855); "Beilstein's Handbuch der Organischen Chemie," Julius Springer, Berlin, 1918, Vol. I, p. 360.

(2) Friedel, A., *Liebigs Ann. Chem.*, **124**, 327(1862); vgl. Kolbe, *Jahresber. fortsch. Chem.*, 405(1862); "Beilstein," *ibid.*, p. 360.

(3) Anchutz, R., "Richter's Organic Chemistry," Vol. 1, P. Blakiston's Son and Co., Philadelphia, 1913, p. 125.

(4) Zouboff, B., *Chem. Zentr.*, **70**, II, 586(1899); "Beilstein," *ibid.*, p. 361.

(5) Heinrich, George, *Chem. Zentr.*, **71**, II, 156(1900); "Beilstein," *ibid.*, p. 361.

(6) Tischtschenko, W., *Chem. Zentr.*, **71**, I, 12(1900); "Beilstein," *ibid.*, p. 362.

(7) Ipatjew, W., *Ber.*, **37**, 2979, 3000(1904); *Chem. Zentr.*, **75**, II, 1020(1904); "Beilstein," *ibid.*, p. 361.

(8) Menschutkin, B., *Chem. Zentr.*, **77**, I, 1869(1906);

Z. anorg. Chem., **52**, 17(1906); "Beilstein," *ibid.*, p. 362.
(9) Ipatjew, W., *Chem. Zentr.*, **78**, II, 2035(1907); "Beilstein," *ibid.*, p. 361.
(10) Guerbet, Marcel, *Chem. Zentr.*, **80**, I, 129(1909); *ibid.*, **80**, II, 1537(1909); "Beilstein," *ibid.*, p. 362.
(11) Schultz, G., *Pharm. J.*, 85, 664(1910); *Ber.*, **43**, 2517 (1910); *Proc. Am. Pharm. Assoc.*, **59**, 412(1911).
(12) Guerbet, Marcel, *Compt. rend.*, **164**, 952–54(1917); *C. A.*, **11**, 2661(1917); **12**, 1383(1918).
(13) Atkins, William R. G., *J. Chem. Soc.*, **117**, 218–20 (1920); *C. A.* **14**, 1967(1920).
(14) Ellis, Carleton, *Petroleum Mag.*, **10**, 40–41(1921); *C. A.*, **15**, 1617–18(1921).
(15) *Chemical Age* (London), **4**, 440–43(1921); *C. A.*, **15**, 2061(1922).
(16) Boruttau, H., *Deut. med. Wochschr.*, **47**, 747(1921); *Index Medicus*, **6**, 522(1921).
(17) *Merck Rept.*, **31**, 5(1922); *Yearbook Am. Pharm. Assoc.*, **11**, 42(1922).
(18) Grant, D. H., and Johns, C. O., *Am. J. Pharm.*, **94** 418(1922); *Yearbook Pharm.*, 268(1922).
(19) Bernhardt, G., *Deut. med. Wochschr.*, **48**, 68–69 (1922); *C. A.*, **17**, 784(1923).
(20) *Perfumery Record*, **14**, 267(1923); *Yearbook Pharm.*, 108(1923).
(21) Ellis, Carleton, U. S. Pat. 1,582,912, May 4, 1926.
(22) Hering, K., *Pharm. Ztg.*, **72**, 966(1927); *Yearbook Am. Pharm. Assoc.*, **16–17**, 378(1927–1928).
(23) Schamelhout, A. J., *J. pharm. Belg.*, **9**, 401(1927); *Yearbook Am. Pharm. Assoc.*, *op. cit.*
(24) Ducommun, F., *Pharm. Acta. Helv.*, **13**, 185–209 (1938); *C. A.*, **33**, 9551(1939).
(25) Marki, W., *Pharm. Acta. Helv.*, **13**, 210–26, 227–70 (1938); *Chimie & Industrie*, **42**, 109(1939); *Pharm. Abstracts*, **6**, 502(1940).
(26) Kunz, Eric C., and Luthy, Max, U. S. Pat. 2,293,551, Aug. 18, 1943; *C. A.*, **37**, 1015(1943).
(27) Chase, J. F., Lehman, A. J., Crandall, E. R., Schwerma, Henry, and Bauer, R. O., THIS JOURNAL, **33**, 109–11(1944).
(28) Harris, Lewis E., THIS JOURNAL, *Pract. Pharm. Ed.*, **5**, 38–40(1944); *C. A.* **38**, 2448(1944).
(29) Clouse, R. C., and Bamford, M. W., *Am. J. Pharm.*, **115**, 261–62(1943); *C. A.*, **38**, 216(1944).
(30) Harris, Lewis E., *Proc. Am. Pharm. Mfgrs. Assoc., Mid-Year meeting*, pp. 34–38(1943); *C. A.*, **38**, 4382(1944).
(31) Burlage, H. M., THIS JOURNAL, *Pract. Pharm. Ed.*, **7**, 209–11(1946).
(32) Shiflett, B. O., *Practical Druggist and Spatula Consolidated*, **48**, 17–18(1930).
(33) La Rocca, Joseph, and Burlage, Henry M., *J. Elisha Mitchell Scientific Soc.*, **61**, Nos. 1–2, 220–27(1945).

Book Review

Goethe's Botany, by AGNES ARBER. The Chronica Botanica Company, Waltham, Mass., and G. E. Stechert and Co., New York, 1946. 63 pages. 17.5 × 26 cm. 6 illus. Price $2.00.

It is significant of the frantic longing of present day mankind for tracing back all the confusing complexity of life to some fundamental bases on which the edifice of an all-embracing coordinated something—a "ONE WORLD"—could be erected, that similar efforts are given renewed attention.

It was in 1790 that the German poet Goethe's *Attempt To Interpret the Metamorphosis of Plants* appeared. Its purpose was to create (and to prove) the concept of a fundamental ["*Ur*"] plant from which the whole body of plants now in existence may be derived. The discussion of this astounding publication has never ceased. It gained, however, a new impulse after the first world war. There have appeared during the last twenty-five years a number of German treatises on this subject and a remarkable English one (by Ch. Sherrington in 1942) and, while there was only one English version (published in 1863) of Goethe's pamphlet until quite recently another one was published in 1937 and, but nine years later, the one under consideration.

The translation by Agnes Arber comes close not only to the letter, but also to the spirit, of Goethe's pamphlet. Her introduction, finally, merits the highest praise possible, namely the statement that it really means what its title says, i.e., that the reader is actually "introduced" into the problem and the attempts at its explanation. The following quotation reveals Miss Arber's idea of Goethe's type concept and its place in Goethe's general philosophy:

"In Goethe's eyes the type principle was the clue to the interpretation of animals as well as plants . . . None of Goethe's thinking was ever isolated from his whole mental activity, and the type concept, or, more widely the idea of *Ur* [fundamental] phenomena, was to him a clue to be followed not in science merely; it was, rather, one of the keys which gave him the freedom of the universe as a whole."

Miss Arber quotes a book by Julius Schuster published in 1924 and entitled *Goethe, die Metamorphose der Pflanzen, mit dem Original bildwerk* (Goethe, the metamorphosis of plants, with the original illustrations). She does not mention the same author's essays on "*Idealistische Morphologie-als Gegenwartsproblem*" (Idealistic Morphology as a Problem of Our Time), published in 1928, and "*Goethe und die Biologie*" (1932). It is the last publication that Schuster gives the following extremely German explanation, which is most significant:

"Goethe's *Ur* [fundamental] phenomenon itself is not a reality anymore, but a visionary abstraction, an abstract picture. This abstraction, however—this generalization—sought by Goethe is by no means identical with the common idea of something general: It still remains something that is individual. And things individual cannot be explained by means of concepts but only by perception—by pictures. Goethe requires that the individual, while brought into line with the continuous sequence of happenings, retain its identity within this order. It must *be* and *remain* individual, if the general shall truly be represented to him. It is the problem of individualizing perception which we are facing here."

In conclusion it may be stated that in the astounding figure of Goethe mankind has been given one of the very rare representatives of the race, human enough to be moved by all the problems of man and great enough to carry them to the highest level of consideration. Thus, on the centenary (1932) of his death there was scarcely one branch of science, art, and thought that did not testify to the influence of Goethe. For pharmacy this was done by this reviewer in an article "*Goethe und die Pharmazie*" [*Pharm. Ztg.*, **77**, 333–39(1932)].—GEORGE URDANG.

AUTHOR INDEX

A

Abramson, H. A., *see* Brown, E. A.
Ahlquist, R. P., the interaction of sympathomimetic pressor amines: inversion of the vonedrine pressor action by paredrine, 348
Anderson, R. C., and Chen, K. K., the colorimetric assay of digitoxin by the modified Raymond Method, 353

B

Bachman, G. B., *see* Wetzel, J. W.
Bandelin, F. J., and Kemp, C. R., the evaluation of various bases for sulfonamide ointments, 65
Barlow, O. W., *see* Hunt, W. H.
Bartels, B., *see* Holck, H. G. O.
Basu, N. K., and Pabrai, P. R., a chemical investigation of *Celastrus paniculata* Willd., 272
—— and Lamsal, P. P., a chemical investigation of *Sphaeranthus indicus* Linn., 274
Beal, G. D., *see* Waters, K. L.
Bell, F. K., and Krantz, J. C., Jr., digitalis. IV. The collaborative study of the assay of digitalis and its preparations by the chemical and cat methods, 260
Bergeim, O., *see* Woodson, H. W.
Blanchard, E. W., *see* Heinemann, B.
Bliss, C. I., a revised cylinder-plate assay for penicillin, 6
Brewer, J. H., a simple pipette washer for the average laboratory, 325
Brimi, R., *see* Levey, S.
Brown, D. J., *see* Seeberg, V. P.
Brown, E. A., Abramson, H. A., Gorin, M. H., Kauffmann, H. O., and Shanley, E. S., the stability of solutions of urea peroxide in glycerol, isopropanol, and ethylene glycol, 304
Buelow, D., *see* Gisvold, O.
Bundy, C. H., and Edman, W. W., investigations of the heavy metals test in the U. S. P. XII, 115
Burlage, H. M., and Hawkins, D. B., pharmaceutical applications of isopropyl alcohol. I. As a solvent in pharmaceutical manufacturing, 379
Busse, L. W., *see* Russell, J. C. H.
Butz, C., *see* Kuna, S.

C

Carlson, E. H., *see* Gisvold, O.
Carol, J., and Rotondaro, F. A., the determination of estrone in oil solutions of estrogens, 176
Chaikelis, A. S., the thiamine content of herbs and medicinal plants, 343
Chen, K. K., *see* Anderson, R. C.
Cheng, Y.-F., Christian, J. E., and Jenkins, G. L., the synthesis of chemotherapeutic agents. III. The synthesis of certain thio compounds, 334
Christian, J. E., *see* Cheng, Y.-F.; Neuroth, M. L.; Wetzel, J. W.
——, Jenkins, G. L., Keagle, L. C., and Crum, J. A., the synthesis of chemotherapeutic agents. I. The synthesis of certain thio and dithio compounds, 328
Ciminera, J. L., and Wilcox, P. W., stable ascorbic acid solution for parenteral use, 363
Collins, W. R., and Kirch, E. R., determination and stability of menadione, 215
Copley, D. M., effect of time and temperature in the preparation of certain fats and waxes for melting point determinations—report no. two, 78; report no. three, 84
Crum, J. A., *see* Christian, J. E.

D

Dietz, E. A., and Folkers, K., erythrina alkaloids. XV. A colorimetric determination of β-erythroidine, 48
Dillon, C. P., Estabrook, G. B., and Hartung, W. H., the influence of physiologically active compounds and their inactive homologs on phase boundary potentials, 250
Dodd, M. C., Hartmann, F. W., and Ward, W. C., surface-active agents as ointment bases, 33

E

Eble, T. E., *see* Mundell, M.
Edison, A. O., *see* Kuna, S.
Edman, W. W., *see* Bundy, C. H.
Edwards, L. D., *see* Haley, T. J.
Eisenberg, W. V., *see* Keenan, G. L.
Estabrook, G. B., *see* Dillon, C. P.

F

Farson, deC., *see* Iwamoto, H. K.
Fischbach, H., *see* Mundell, M.
Fischelis, R. P., a review of the present status of barbiturate regulation, 193
Fischer, L., *see* Miller, O. H.
Folkers, K., *see* Dietz, E. A.
Fosbinder, R. J., *see* Hunt, W. H.
Foster, C. P., *see* Hartung, W. H.
Foter, M. J., *see* Quisno, R. A.
Frederick, M., and Koff, A., the determination of phenolphthalein in mineral oil emulsions, 158

G

Gakenheimer, W. C., and Reguera, R. M., an assay for choline chloride in pharmaceutical products, 311
Geiger, E., Schnakenberg, G., and Geiger, L. A., action of cholic-, desoxycholic-, dehydrocholic-, and dehydrodesoxycholic acids on body temperature in rabbits, 73
Geiger, L. A., *see* Geiger, E.
Gershberg, S., and Stoll, F. D., apparatus for tablet disintegration and for shaking-out extractions, 284
Gibby, I. W., *see* Quisno, R. A.
Giese, A. C., and Wells, J. M., sweat- and water-resistant sunburn preparations, 208
Gisvold, O., Buelow, D., and Carlson, E. H., the synthesis of some alpha, omega-bis-(3,4-dihydroxyphenyl) alkanes, 188
Golden, M. J., peroxide test for mineral oils, 76
Goldstein, S. W., determination of magnesium in magnesium sulfate and solution of magnesium citrate, 19
Gorin, M. H., *see* Brown, E. A.
Green, M. W., some modern contributions to the enzyme theory of pharmacology, 1; *see* Martin, M. E.
Gross, E. G., *see* Maney, P. V.
Grote, I. W., and Walker, P., studies in the preservation of liquid pharmaceutical preparations. I. The relationship between the preservative action of alcohol and the solid content of the preparation, 182

H

Haden, H. H., Jr., *see* Hadley, W. J.
Hadley, W. J., and Haden, H. H., Jr. Alabama's "sheep kill" laurel, *Kalmia latifolia* L.—a preliminary study, 340
Haley, T. J., and Edwards, L. D., a drop-counter for saline solutions, 53
——, Edwards, L. D., and Johnson, C. H., a chemical study of two isomeric sodium hydroxymercuribenzoates, 179
Hartmann, F. W., *see* Dodd, M. C.
Hartung, W. H., *see* Dillon, C. P.; Simonoff, R.
—— and Foster, C. P., aminoalcohols. XV. *p*-Aminopropadrine, 15
Hazleton, L. W., and Hellerman, R. C., the influence of vehicles on the action of drugs, 161
—— and Riley, A. S., studies on the pharmacology of aluminum cholate, 213
Heinemann, B., and Blanchard, E. W., properties of a new mercurial antiseptic, 2-acetoxymercuri-4-diisobutylphenol, 54
Hellerman, R. C., *see* Hazleton, L. W.
Henderson, J., the status of tyrothricin as an antibiotic agent for topical application, 141
Hilty, W. W., *see* Wilson, D. T.
Hirose, R. S., *see* Huyck, C. L.
Hoehn, W. M., and Johnson, R., the toxicity of sodium 3,13-dihydroxy-7-cholanate and its influence on the bile flow in rats, 296
Holck, H. G. O., Kimura, K. K., and Bartels, B., effect of the anesthetic and the rate of injection of digitalis upon its lethal dose in cats, 366
Holt, K. E., the extraction of caffeine from pharmaceutical preparations, 313; the separation of acetylsalicylic acid, acetophenetidin, and caffeine, 71
Hoyt, R. E., *see* Levine, M. G.
Hueper, W. C., *see* Ichniowski, C. T.
Hunt, W. H., Fosbinder, R. J., and Barlow, O. W., the anesthetic effects and toxicities of some new barbituric acid derivatives administered to mice and rats, I, 231; administered to rabbits, II, 244
Huyck, C. L., Hirose, R. S., and Reyes P. A., Jr., diffusion of sulfonamides from emulsified ointment bases, 129

I

Ichniowski, C. T., and Hueper, W. C., pharmacological and toxicological studies on salicylamide, 225
Illg, P. L., *see* Seeberg, V. P.
Iwamoto, H. K., and Farson, deC., a contribution to the chemistry and pharmacology of glycolanilide, 50

J

Jenkins, G. L., *see* Cheng, Y.-F.; Christian, J. E.; Neuroth, M. L.; Wetzel, J. W.
Johnson, C. H., *see* Haley, T. J.
Johnson, F. F., *see* Seeberg, V. P.
Johnson, R., *see* Hoehn, W. M.
Jones, J. W., *see* Maney, P. V.

K

Kauffmann, H. O., *see* Brown, E. A.
Keagle, L. C., *see* Christian, J. E.
Keenan, G. L., the microscopic identification of demerol, 338
—— and Eisenberg, W. V., tables for the microscopic identification of N. F. VII crystalline substances, 94
Kelly, K. L., *see* Martin, M. E.
Kemp, C. R., *see* Bandelin, F. J.
Kimura, K. K., *see* Holck, H. G. O.
Kirch, E. R., *see* Collins, W. R.; Woodson, H. W.
Knudsen, L., *see* Welch, H.
Koff, A., *see* Frederick, M.
Koppanyi, T., *see* Vivino, A. E.
Korns, H. M., *see* Maney, P. V.
Krantz, J. C., Jr., *see* Bell, F. K.
Kuna, S., Edison, A. O., and Butz, C., a method for large-scale testing for pyrogens, 59

L

Lamsal, P. P., *see* Basu, N. K.
Lasichak, A. G., *see* Levey, S.
Lazier, W. A., *see* Mattocks, A. M.
Lee, C. O., *see* Neuroth, M. L.
Levey, S., Lasichak, A. G., Brimi, R., Orten, J. M., Smyth, C. J., and Smith, A. H., a study to determine the toxicity of fumaric acid, 298
Levine, M. G., and Hoyt, R. E., persistence of penicillin in saliva after local oral application, 346
Ligon, E. W., Jr., *see* Way, E. L.
Lyster, S. C., *see* Nelson, J. W.

M

McDaniel, G. E., *see* Yeager, O. W.
Maney, P. V., Jones, J. W., Gross, E. G., and Korns, H. M., dihydroxypropyl theophylline: its preparation and pharmacological and clinical study, 266
Martin, M. E., Kelly, K. L., and Green, M. W., a study of the determination of some official aldehydes, 220
Mattocks, A. M., and Lazier, W. A., hydrophilic acid ointments for the debridement of burns, 275

Miller, O. H., and Fischer, L., comparative analyses of normal and tetraploid *Datura stramonium* and *Datura tatula*, 23
Mildworm, L., *see* Rosenblum, H.
Molitor, H., Gundel, M. E., Kuna, S., and Ott, W. H., studies on pyrogens. II. Some factors influencing the evaluation of pyrogen tests, 356
Moran, W. J., *see* Puetzer, B.
Mundell, M., Fischbach, H., and Eble, T. E., the chemical assay of penicillin, 373

N

Nelson, J. W., and Lyster, S. C., the toxicity of myristyl-gamma-picolinium chloride, 89
Neuroth, M. L., Lee, C. O., Christian, J. E., and Jenkins, G. L., a study of penicillin ointments and creams, 321

O

Oneto, J. F., the alkaloids of species of Garrya. I. Isolation of alkaloids, 204
Orland, F. J., and Orland, P. M., pentobarbital sodium anesthesia in the Syrian hamster, 263
Orland, P. M., *see* Orland, F. J.
Orten, J. M., *see* Levey, S.

P

Pabrai, P. R., *see* Basu, N. K.
Price, C. W., *see* Welch, H.
Puetzer, B., and Moran, W. J., separation of *l*-menthol from racemic menthol U. S. P., 127

Q

Quisno, R. A., Gibby, I. W., and Foter, M. J., the effect of agar upon the germicidal efficiency of the quaternary ammonium salts, 317

R

Radomski, J. L., and Woodard, G., a survey of the present status of red squill as a rodenticide, 289
Randall, W. A., *see* Welch, H.
Reguera, R. M., *see* Gakenheimer, W. C.
Reider, R. F., *see* Yeager, O. W.
Reyes, P. A., Jr., *see* Huyck, C. L.
Richards, R. K., *see* Spielman, M. A.
Riley, A. S., *see* Hazleton, L. W.
Rosenblum, H., and Mildworm, L., quantitative determination of saccharin, 336
Rosin, J., U. S. P. melting point reference standards, 56
Rotondaro, F. A., *see* Carol, J.
Russell, J. C. H., Busse, L. W., and Uhl, A. H., studies on the cutaneous absorption of insulin, 217

S

Schnakenberg, G., *see* Geiger, E.
Seeberg, V. P., Brown, D. J., and Johnson, F. F., stability of penicillin in solutions of ephedrine, 326
——, Illg, P. L., Brown, D. J., and Wickert, M. L., the gastrointestinal absorption and destruction of penicillin, 280
Shanley, E. S., *see* Brown, E. A.
Simonoff, R., and Hartung, W. H., amino alcohols. XVII. Arylethanolamines, 306
Smith, A. H., *see* Levey, S.
Smyth, C. J., *see* Levey, S.
Soine, T. O., the β-hydroxy and β-halo ethyl 3- and 4-nitro phthalimides, 42
Sperling, H. G., *see* Wilson, C. O.
Spielman, M. A., and Richards, R. K., the analgesic properties of some 1-acyl-3-aminomethylureas, 28
Stoll, F. D., *see* Gershberg, S.

U

Uhl, A. H., *see* Russell, J. C. H.

V

Vivino, A. E., and Koppanyi, T., the effect of continued oral administration of lauric and myristic acid ester of colaminoformylmethylpyridinium chloride (emulsept) to the albino rat, 169

W

Walker, P., *see* Grote, I. W.
Ward, W. C., *see* Dodd, M. C.
Warren, M. R., and Werner, H. W., the evaluation of antipyretics against pyrogen-induced fever, 257
Waters, K. L., and Beal, G. D., notes on the water content of crystalline caffeine, 12
Way, E. L., studies on the local anesthetic properties of isonipecaine, 44
—— and Ligon, E. W., Jr., some cardiac effects of isonipecaine (demerol), 113
Welch, H., Price, C. W., and Randall, W. A., increase in fatality rate of *E. typhosa* for white mice by streptomycin, 155
——, Randall, W. A., and Knudsen, L., methods of testing antibiotic substances and limitations involved, 102
Weldton, D. E., *see* Wetzel, J. W.
Wells, J. M., *see* Giese, A. C.
Werner, H. W., *see* Warren, M. R.
Wetzel, J. W., Weldton, D. E., Christian, J. E., Jenkins, G. L., and Bachman, G. B., the synthesis of chemotherapeutic agents. II. The synthesis of certain thio and dithio compounds, 331
Wickert, M. L., *see* Seeberg, V. P.
Wilcox, P. W., *see* Ciminera, J. L.
Wilson, C. O., and Sperling, H. G., determination of zinc oxide and bentonite in calamine lotion, 174
Wilson, D. T., and Hilty, W. W., the assay of medicinal compounds having caffeine as a common ingredient, 97
Woodard, G., *see* Radomski, J. L.
Woodson, H. W., Kitch, E. R., and Bergeim, O., a study of the microbiological assay of riboflavin, 253

Y

Yeager, O. W., Reider, R. F., and McDaniel, G. E., evaluation of totaquine in treatment of malaria in an endemic malarious area, 337
Youngken, H. W., studies on Indian rhubarb. II. *Rheum emodi*, 148

SUBJECT INDEX

A

Acetophenetidin, acetylsalicylic acid, and caffeine, the separation of, 71
2-Acetoxymercuri-4-diisobutylphenol, a new mercurial antiseptic, properties of, 54
Acetylsalicylic acid, acetophenetidin, and caffeine, the separation of, 71
1-Acyl-3-aminomethylureas, the analgesic properties of some, 28
Agar, the effect of, upon the germicidal efficiency of the quaternary ammonium salts, 317
Alcohol, the relationship between the preservative action of, and the solid content of the preparation, I. Studies in the preservation of liquid pharmaceutical preparations, 182
Aldehydes, a study of the determination of some official, 220
Alkaloids, erythrina. XV. A colorimetric determination of β-erythroidine, 48
isolation of, I. Alkaloids of species of Garrya, 204
Alkanes, the synthesis of some *alpha, omega*-bis-(3,4-dihydroxyphenyl), 188
Aluminum cholate, studies on the pharmacology of, 213
Amino alcohols. XV. *p*-Aminopropadrine, 15; XVII. Arylethanolamines, 306
Aminomethylureas, analgesic properties of some 1-acyl-3-, 28
***p*-Aminophenylalanine,** a synthesis of, 18
***p*-Aminopropadrine,** XV. Aminoalcohols, 15
Ammonium salts, quaternary, the effect of agar upon the germicidal efficiency of the, 317
Analgesic properties of some 1-acyl-3-aminomethylureas, 28
Anesthesia, pentobarbital sodium, in the Syrian hamster, 263
Anesthetic, effect of the, and the rate of injection of digitalis upon its lethal dose in cats, 366
effects and toxicities of some new barbituric acid derivatives administered to mice and rats, I, 231; II. Administered to rabbits, 244
properties, local, of isonipecaine, studies on the, 44
Antibiotic agent, the status of tyrothricin as an, for topical application, 141
Antibiotic substances, methods of testing, and limitations involved, 102
Antipyretics, the evaluation of, against pyrogen-induced fever, 257
Antiseptic, properties of a new mercurial, 2-acetoxymercuri-4-diisobutylphenol, 54
Apparatus for tablet disintegration and for shaking-out extractions, 284
Arylethanolamines, XVII. Amino alcohols, 306
Ascorbic acid solution, stable, for parenteral use, 363

B

Barbiturate regulation, a review of the present status of, 193
Barbituric acid derivatives, the anesthetic effects and toxicities of some new, administered to mice and rats, I, 231; II. Administered to rabbits, 244
Bentonite and zinc oxide, determination of, in calamine lotion, 174
Bile flow, in rats, the toxicity of sodium 3,12-dihydroxy-7 cholanate and its influence on, 296
Book Reviews
Acetanilid—A Critical Bibliographic Review, 351
American Pharmacy, 255
The Chemistry of Acetylene, 319
Colloids—Their Properties and Applications, 352
Esquisse De Mes Voyages Au Bresil Et Paraguay, 320
Goethe's Botany, 384
Hayfever Plants, 64
Introduction to Emulsions, 352
Major Instruments of Science and Their Applications to Chemistry, 32
New Drugs, 191
Official and Tentative Methods of Analysis, 224
Organic Qualitative Microanalysis, 352
Physical Chemistry of Cells and Tissues, 64
Physical Constants of Hydrocarbons. Vol. III, Mononuclear Aromatic Hydrocarbons, 320
Physical Methods of Organic Chemistry, Vol. I, 32
Physical Methods of Organic Chemistry, Vol. II, 351
Richter's Chemistry of the Carbon Compounds, 351
Scientific Societies in the United States, 287
Soil and Plant Analysis, 192
Surface Active Agents, 187
Textbook of Pharmacognosy, 320
Vitamins and Hormones, 160
Burns, hydrophilic acid ointments for the debridement of, 275

C

Caffeine, acetophenetidin, and acetylsalicylic acid, the separation of, 71
as a common ingredient, the assay of medicinal compounds having, 97
the extraction of, from pharmaceutical preparations, 313
notes on the water content of crystalline, 12
Calamine lotion, determination of zinc oxide and bentonite in, 174
Cardiac effects, some, of isonipecaine (demerol), 113
Celastrus paniculata Willd., a chemical investigation of, 272
Chemical assay of penicillin, 373
Chemical investigation of *Celastrus paniculata* Willd., 272
of *Sphaeranthus indicus* Linn., 274
Chemotherapeutic agents, the synthesis of. I. The synthesis of certain thio and dithio compounds, 328; II. The synthesis of certain thio and dithio compounds, 331; III. The synthesis of certain thio compounds, 334
Cholanate, sodium 3,12-dihydroxy-7, the toxicity of, and its influence on bile flow in rats, 296
Cholate, aluminum, studies on the pharmacology of, 213
Cholic acid, desoxycholic-, dehydrocholic-, and dehydrodesoxycholic acids, action of, on body temperature in rabbits, 73
Choline chloride, an assay for, in pharmaceutical products, 311
Colaminoformylmethylpyridinium chloride (emulsept), the effect of continued oral administration of lauric and myristic acid ester of, to the albino rat, 169
Colorimetric assay of digitoxin by the modified Raymond method, 353
Creams and ointments, penicillin, a study of, 321

D

Datura stramonium and *Datura tatula*, comparative analyses of normal and tetraploid, 23
Dehydrocholic acid, cholic-, desoxycholic-, and dehydrodesoxycholic acids, action of, on body temperature in rabbits, 73
(Demerol) isonipecaine, some cardiac effects of, 113
the microscopic identification of, 338
Desoxycholic acid, cholic-, dehydrocholic-, and dehydrodesoxycholic acids, action of, on body temperature in rabbits, 73
Digitalis. IV. The collaborative study of the assay of digitalis and its preparations by the chemical and cat methods, 260
effect of the anesthetic and the rate of injection of, upon its lethal dose in cats, 366
Digitoxin, colorimetric assay of, by the modified Raymond method, 353
alpha, omega-**bis-(3,4-Dihydroxyphenyl) alkanes,** the synthesis of some, 188
Dihydroxypropyl theophylline: its preparation and pharmacological and clinical study, 266
Dithio and thio compounds, the synthesis of certain, I and II. The synthesis of chemotherapeutic agents, 328, 331
Drop-counter for saline solutions, 53
Drugs, the influence of vehicles on the action of, 161

E

(Emulsept) colaminoformylmethylpyridinium chloride, the effect of continued oral administration of lauric- and myristic acid ester of, to the albino rat, 169
Emulsified ointment bases, diffusion of sulfonamides from, 129
Emulsions, mineral oil, the determination of phenolphthalein in, 158
Enzyme theory of pharmacology, some modern contributions to the, I
Ephedrine, stability of penicillin in solutions of, 326
Erythrina alkaloids. XV. A colorimetric determination of β-erythroidine, 48
β-Erythroidine, a colorimetric determination of, XV. Erythrina alkaloids, 48
Estrogens, the determination of estrone in oil solutions of, 176
Estrone, the determination of, in oil solutions of estrogens, 176
Ethanolamines, aryl, XVII. Amino alcohols, 306

F

Fats and waxes, effect of time and temperature in the preparatiin of certain, for melting point determinations—report no. two, 78; report no. three, 84
Fertilizers, the influence of, on the growth and alkaloidal content of *Hyoscyamus niger* Linn., 121
Fumaric acid, a study to determine the toxicity of, 298

G

Garrya, the alkaloids of species of. I. Isolation of alkaloids, 204
Germicidal efficiency of the quaternary ammonium salts, the effect of agar upon the, 317
Glycolanilide, a contribution to the chemistry and pharmacology of, 50

H

Heavy metals test in the U. S. P. XII, investigations of the, 115
Herbs and medicinal plants, thiamine content of, 343
Hydrophilic acid ointments for the debridement of burns, 275
β-Hydroxy and β-halo ethyl 3- and 4-nitro phthalimides, 42
Hydroxymercuribenzoates, sodium, a chemical study of two isomeric, 179
Hyoscyamus niger Linn., the influence of fertilizers on the growth and alkaloidal content of, 121

I

Indian rhubarb, studies on. II. *Rheum emodi*, 148
Insulin, studies on the cutaneous absorption of, 217
Isonipecaine (demerol), some cardiac effects of, 113
studies on the local anesthetic properties of, 44
Isopropyl alcohol, pharmaceutical applications of. I. As a solvent in pharmaceutical manufacturing, 379

K

Kalmia latifolia L., Alabama's "sheep kill" laurel—a preliminary study, 340

L

Lauric- and myristic acid ester of colaminoformylmethylpyridinium chlo-

ride, (emulsept) the effect of continued oral administration of, to the albino rat, 169
Liquid pharmaceutical preparations, studies in the preservation of. I. The relationship between the preservative action of alcohol and the solid content of the preparation, 182

M

Magnesium, determination of, in magnesium sulfate and solution of magnesium citrate, 19
Magnesium citrate, solution of, and magnesium sulfate, determination of magnesium in, 19
Magnesium sulfate and solution of magnesium citrate, determination of magnesium in, 19
Malaria, evaluation of totaquine in treatment of, in an endemic malarious area, 337
Medicinal plants and herbs, thiamine content of, 343
Melting point determinations, effect of time and temperature in the preparation of certain fats and waxes for—report no. two, 78; report no. three, 84
Melting point reference standards, U. S. P., 56
Menadione, determinatiin and stability of, 215
l-**Menthol,** separation of, from racemic menthol U. S. P., 121
Mercurial antiseptic, properties of a new, 2-acetoxymercuri-4-diisobutylphenol, 54
Microbiological assay of riboflavin, a study of the, 253
Microscopic identification of N. F. VII crystalline substances, tables for the, 94
Mineral oil emulsions, the determination of phenolphthalein in, 158
Mineral oils, peroxide test for, 76
Myristic- and lauric acid ester of colaminoformylmethylpyridinium chloride, (emulsept) the effect of continued oral administration of, to the albino rat, 169
Myristyl-gamma-picolinium chloride, the toxicity of, 89

N

N. F. VII crystalline substances, tables for the microscopic identification of, 94
Nitro phthalimides, 3- and 4-, β-hydroxy and β-halo ethyl, 42

O

Ointment bases, emulsified, diffusion of sulfonamides from, 129
surface-active agents as, 33
Ointments and creams, penicillin, a study of, 321
hydrophilic acid, for the debridement of burns, 275
sulfonamide, the evaluation of various bases for, 65

P

Paredrine, inversion of the vonedrine pressor action by: the interaction of sympathomimetic pressor amines, 348
Penicillin, the chemical assay of, 373
the gastrointestinal absorption and destruction of, 280
ointments and creams, a study of, 321
persistence of, in saliva after local oral application, 346
a revised cylinder-plate assay for, 6
stability of, in solutions of ephedrine, 326
Pentobarbital sodium anesthesia in the Syrian hamster, 263
Peroxide test for mineral oils, 76
Pharmaceutical preparations, liquid, studies in the preservation of. I. The relationship between the preservative action of alcohol and the solid content of the preparation, 182
Phase boundary potentials, the influence of physiologically active compounds and their inactive homologs on, 250
Phenolphthalein, the determination of, in mineral oil emulsions, 158
Pipette washer, a simple, for the average laboratory, 325
Preservation of liquid pharmaceutical preparations, studies in the. I. The relationship between the preservative action of alcohol and the solid content of the preparation, 182
Pyrethrum culture investigations in Nebraska, 309
Pyrogen-induced fever, the evaluation of antipyretics against, 257
Pyrogens, a method for large-scale testing for, 59
studies on. II. Some factors influencing the evaluation of pyrogen tests, 356

Q

Quaternary ammonium salts, the effect of agar upon the germicidal efficiency of the, 317

R

Raymond method, modified, colorimetric assay of digitoxin by the, 353
Red squill, a survey of the present status of, as a rodenticide, 289
Reference standards, U. S. P. melting point, 56
Regulation, barbiturate, a review of the present status of, 193
Rheum emodi, studies on Indian rhubarb, II, 148
Rhubarb, Indian, studies on. II. *Rheum emodi,* 148
Riboflavin, a study of the microbiological assay of, 253
Rodenticide, a survey of the present status of red squill as a, 289

S

Saccharin, quantitative determination of, 336
Salicylamide, pharmacological and toxicological studies on, 225
Saline solutions, a drop-counter for, 53
"Sheep kill" laurel, Alabama's, *Kalmia latifolia* L.—a preliminary study, 340
Sodium 3,12-dihydroxy-7 cholanate, the toxicity of, and its influence on bile flow in rats, 296
Sodium hydroxymercuribenzoates, a chemical study of two isomeric, 179
Solvent in pharmaceutical manufacturing, I. Pharmaceutical applications of isopropyl alcohol, 379
Sphaeranthus indicus Linn., a chemical investigation of, 274
Squill, red, a survey of the present status of, as a rodenticide, 289
Stramonium, Datura, and Datura tatula, comparative analyses of normal and tetraploid, 23
Streptomycin, increase in fatality rate of *E. typhosa* for white mice by, 155
Sulfonamide ointments, the evaluation of various bases for, 65
Sulfonamides, diffusion of, from emulsified ointment bases, 129
Sunburn preparations, sweat- and water-resistant, 208
Surface-active agents as ointment bases, 33
Sympathomimetic pressor amines, the interaction of: inversion of the vonedrine pressor action by paredrine, 348

T

Tablet disintegration, apparatus for, and for shaking-out extractions, 284
Theophylline, dihydroxypropyl: its preparation and pharmacological and clinical study, 266
Thiamine content of herbs and medicinal plants, 343
Thio compounds, the synthesis of certain, III. The synthesis of chemotherapeutic agents, 334
and dithio compounds, the synthesis of certain, I and II. The synthesis of chemotherapeutic agents, 328, 331
Totaquine, evaluation of, in treatment of malaria in an endemic malarious area, 337
Tyrothricin, the status of, as an antibiotic agent for topical application, 141

U

United States Pharmacopœial Convention, sixth annual financial report, 370
Urea peroxide, the stability of solutions of, in glycerol, isopropanol, and ethylene glycol, 304
U. S. P. XII, investigations of the heavy metals test in the, 115
U. S. P. melting point reference standards, 56

V

Vehicles, the influence of, on the action of drugs, 161
Viburnum prunifolium, the identification of an acid in the root bark of, 315
Vonedrine pressor action, inversion of the, by paredrine: the interaction of sympathomimetic pressor amines, 348

W

Waxes and fats, effect of time and temperature in the preparation of certain, for melting point determinations—report no. two, 78; report no. three, 84

Z

Zinc oxide and bentonite, determination of, in calamine lotion, 174

CHEMICAL LIBRARY

JAN 31 1947

SCIENTIFIC EDITION

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

Vol. XXXV, No. 12 December, 1946

CONTENTS OF THIS ISSUE

The Colorimetric Assay of Digitoxin by the Modified Raymond Method 353
Robert C. Anderson and K. K. Chen

Studies on Pyrogens. II. Some Factors Influencing the Evaluation of Pyrogen Tests 356
Hans Molitor, Mary E. Gundel, Samuel Kuna, and Walther H. Ott

Stable Ascorbic Acid Solution for Parenteral Use 363
Joseph L. Ciminera and Paul W. Wilcox

Effect of the Anesthetic and the Rate of Injection of Digitalis upon Its Lethal Dose in Cats 366
Harald G. O. Holck, Kazuo K. Kimura, and Barbara Bartels

United States Pharmacopœial Convention Sixth Annual Financial Report 370

The Chemical Assay of Penicillin 373
Merlin Mundell, Henry Fischbach, and Thomas E. Eble

Pharmaceutical Applications of Isopropyl Alcohol. I. As a Solvent in Pharmaceutical Manufacturing 379
Henry M. Burlage and Doris Bullard Hawkins

Book Review 384

Index to Journal for 1946 385–390

Index to Pharmaceutical Abstracts for 1946 353–419

Advertising—Cover pages 2, 3, 4 and pp. II to VIII

Justin L. Powers, Editor

Committee on Publications

George D. Beal, *Chairman*
B. V. Christensen
Roy B. Cook
Robert P. Fischelis
Hugo H. Schaefer

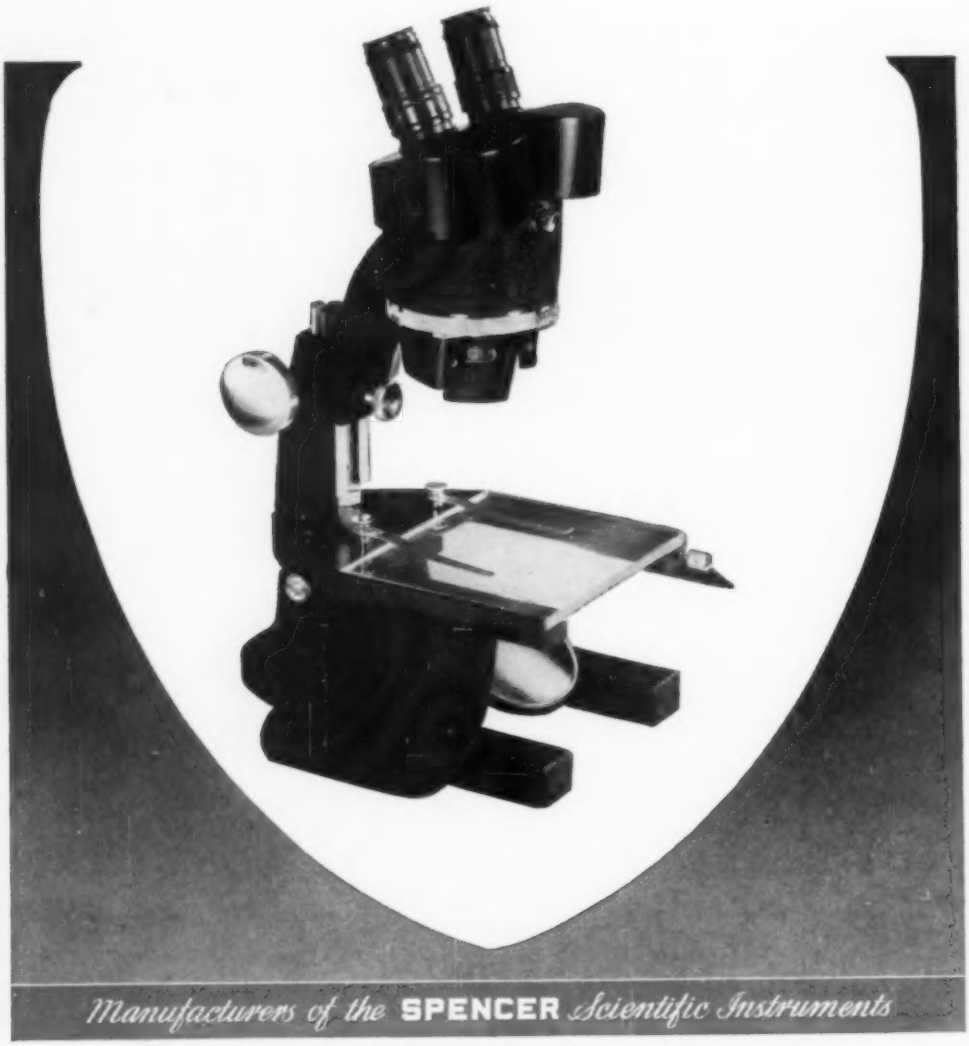
Editorial Advisory Board

T. C. Daniels
L. W. Hazleton
E. J. Hughes
C. O. Lee
W. T. Sumerford
E. H. Wirth

A MICROSCOPE FOR EACH EYE

Spencer Stereoscopic Microscopes provide a complete microscope optical system for each eye, thereby creating vivid depth perception. They are noted for *large field . . . brilliant resolution . . . great depth of focus.* Magnifications range from 6x to 144x. For descriptive literature on Spencer Stereoscopic Microscopes wtite Dept. M107.

American Optical
COMPANY
Scientific Instrument Division
Buffalo 15, New York



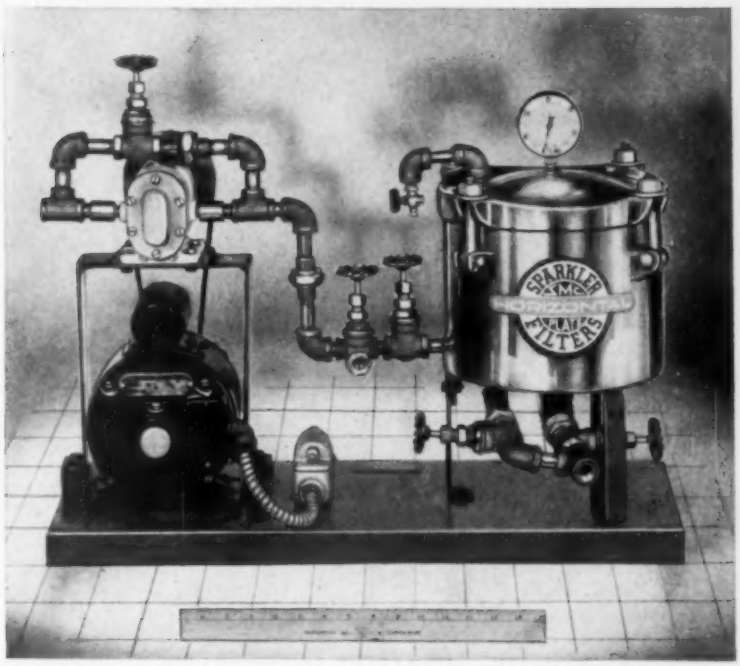
Manufacturers of the **SPENCER** *Scientific Instruments*

PURITY *all the way*

with Sparkler HORIZONTAL PLATE
LIQUID FILTERS

Laboratory Filter in Stainless Steel

Capacity:
1 Pt. to 50 G. P. H.



Basic design is only one of the reasons why Sparkler Filters are in use by the country's leading pharmaceutical firms on such crucially important products as penicillin and intravenous solutions. These are other reasons:

A higher percentage of recovery of valuable liquids is certain with Sparkler's patented Scavenger Plate. This integral part of a Sparkler Filter acts virtually as an auxiliary filter, filtering the very last of each batch. This feature also facilitates blowing cakes dry with gas or air, or washing with solvents.

The evenly built, horizontal cakes retain uniform porosity and produce a high-quality filtrate to the end of each cycle. They cannot slip or break during intermittent operation.

Horizontal plates permit most efficient use of all types of filter media, including germ-proofing discs.

Made in mild steel, stainless, bronze, rubber-lined and many other constructions.

Sparkler Mfg. Co.

381 Lake St. Mundelein, Illinois

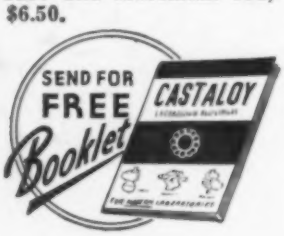
SPARKLER HORIZONTAL PLATE FILTERS

HIGH QUALITY FILTRATION COSTS NO MORE

CASTALOY
LABORATORY APPLIANCES

The Castaloy Burette Holder illustrated above is a widely-used, ingenious device which grips burettes firmly yet obscures no graduations.

Modern Burette Support, including burette holder, with porcelain base and aluminum rod, **$6.50.**



30 different appliances . . . . one for every laboratory need

Castaloy Appliances are superior to ordinary hardware because they cannot rust, they resist corrosion and each one embodies features suiting it to a particular need. Castaloy (Reg. U. S. Pat. Off.) Appliances combine convenience, utility and enduring service.

Manufacturers—Distributors

FISHER SCIENTIFIC CO.
717 Forbes St., **Pittsburgh (19), Pa.**
2109 Locust St., **St. Louis (3), Mo.**

EIMER AND AMEND
Greenwich and Morton Streets
New York (14), New York

Headquarters for Laboratory Supplies